Toxin Reviews, 24:79–94, 2005

ISSN: 0731-3837 print / 1525-6057 online
DOI: 10.1081/TXR-200046403

ANTIVENOM DEVELOPMENT IN AUSTRALIA

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Brown snakes, Pseudonaja *genus, cause more bites and deaths to animals and humans in Australia than any other terrestrial snake genus. Some aspects of treatment of brown snakebites with antivenom remain poorly managed due to the apparent inability of the antivenom to counter the prothrombin activator in the venom. We present evidence of a new novel antivenom (Antiven Pty Ltd Brown snake antivenom (ABSAV)), which shows high efficacy in reversing the hemostatic abnormality caused by brown snake envenomation. It is also more efficient at reversing overall toxicity. In clotting tests, it is substantially more potent than Commonwealth Serum Laboratories Ltd (CSL) Brown snake antivenom and is twice as effective in reversing overall toxicity than CSL Brown snake antivenom.*

Antiven Pty. Ltd. Brown snake antivenom is currently being assessed by the National Registration Authority (veterinary regulative body in Australia).

Keywords: Antivenom, *Pseudonaja*, Elapid, IgY, IgG.

Introduction

There are seven species of brown snakes distributed throughout the Australian mainland. They are Pseudonaja textilis, Pseudonaja nuchalis, Pseudonaja affinis, Pseudonaja inframacula, Pseudonaja ingrami, Pseudonaja guttata, Pseudonaja modesta, and two recognized subspecies of Pseudonaja affinis on offshore islands of Western Australia. Pseudonaja textilis venom has been the most extensively studied venom of the Pseudonaja genus showing only subtle differences. In preliminary studies (Williams et al., 1994), no significant differences could be found in the clotting factors

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TABLE 1 Brown snake venom (South Australia and Queensland) 1 mg/mL, 0.1mL of venom was mixed with 0.1 mL of antivenom dilutions and incubated at 37°C for 30 min. Control: 0.1 mL of venom was mixed with 0.1 mL of saline and incubated at 37°C for 30 min.

Brown snake Antivenoms	Antivenom dilutions with normal saline	Clotting time (seconds)
	Control (venom only)	17
Antiven Pty Ltd	1:4	45
	1:2	83
	undiluted	315
CSL Ltd	1:4	26
	1:2	32
	undiluted	38

in P. textilis, P. nuchalis, P. affinis, or P. inframacula venoms, and of all Pseudonaja bites, the vast majority are from these four species. In fact, there have been no recorded bites from Pseudonaja guttata, the small species of Pseudonaja modesta, or the subspecies of P. affinis, and only a single published account (Brimacombe and Murray, 1995) of a bite from Pseudonaja ingrami Table 1.

In the past, snakebite in Australia has been dominated by tiger snake (*Notechis scutatus*) bites (Mirtschin et al., 1999). More recently, probably due to environmental changes in southern Australia, most snakebites to humans and animals in Australia are due to the brown snake genus *Pseudonaja* (Mirtschin et al., 1998; Sutherland, 1992). While CSL Ltd antivenoms have saved many lives, persistent difficulties are being experienced with its inability to efficiently reverse the effects of the prothrombin activator (Sprivulis et al., 1996; Tibbals et al., 1992). Unrelenting coagulopathy due to the slow reversal of the prothrombin activator presents the added risk of cerebral hemorrhage to the victim (Sutherland, 1995). There has been little research so far that has led to any alterations to the brown snake antivenom to counter the efficacy problem Figures 1, 2, 3, 4, 5, 6 and 7.

Coupled with the poor functioning of CSL Ltd brown snake antivenom at reversing the procoagulant in *Pseudonaja* venoms, it has long been the belief that the *Pseudonaja* genus were low venom yielding snakes. Recent work has shown that the venom

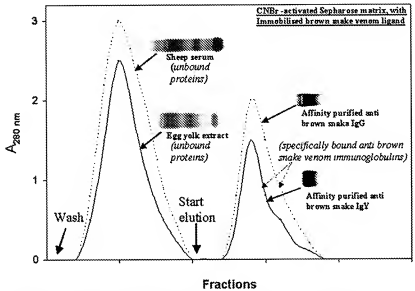


FIGURE 1 Affinity chromatography purification of antibrown snake venom immunoglobin from egg yolk extract and hyperimmune sheep serum.

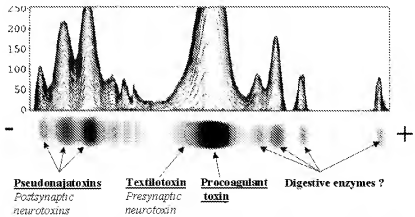
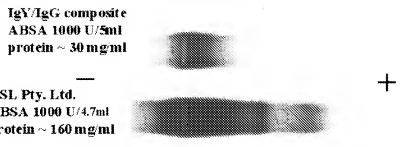


FIGURE 2 Agarose gel electrophoresis and quantitative protein scan of *Pseudonaja textilis* venom. The major components are shown. The presynaptic neurotoxin, textilotoxin, and the prothrombin activator are negatively charged and the small molecular weight postsynaptic neurotoxins are positively charged. The quantitative protein scan shows that the major venom component is the procoagulant toxin and makes up about 40% of the total venom proteins.



Comparative agarose gel electrophoresis of IgY/IgG composite ABSA and CSL Pty., Ltd.ABSA.

FIGURE 3 Protein pattern of brown snake antivenoms. The affinity purified IgY/IgG antivenom contains much less protein for the same units of activity. In the CSL (F(ab')2) antivenom, the fast moving proteins close to the + ve electrode are probably undigested IgG Fc fragments .

yields of most *Pseudonaja* species and geographical variants are significantly more than previously thought (Masci et al., 1998). This finding has provided an even greater impetus to find new ways to improve antivenoms to cope with the problem of reversing the procoagulant.

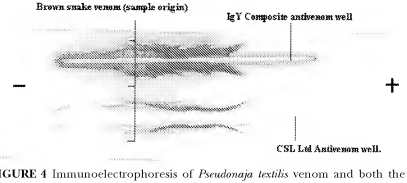


FIGURE 4 Immunoelectrophoresis of *Pseudonaja textilis* venom and both the composite antivenom and CSL brown snake antivenom. When compared to Fig. 2, the composite IgY/IgG antivenom shows strong affinity to all toxins and generally reacts stronger than that of CSL brown snake antivenom. (Both antivenoms had 1000 U/vial activity.)

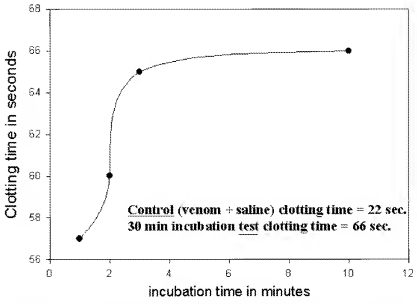


FIGURE 5 The kinetics of venom procoagulant inhibition by the IgV/IgG composite brown 100 μL of brown snake venom (1 mg/mL) was mixed with 100 μL of ABSA (protein concentration 12 mg/mL) and incubated at 37°C. At various time intervals an aliquot was withdrawn and a clotting assay performed. The results show that the antibodies very rapidly neutralize the venom procoagulant toxin, and in the assay conditions the bulk of the toxin is neutralized in about 5 minutes.

Materials and Methods

Materials

All the reagents used in the antivenom manufacture were of analytical grade, and the water used in making up the reagents was sterile, nonpyrogenic (Baxter Healthcare Pty. Ltd.) All solution-reagent transfers were done in a Class II Biological Hazard Cabinet, or in a "Class 350 Clean Room."

ANTIVENOMS

CSL Brown Snake antivenom was purchased from CSL Ltd. Parkville, Victoria, Australia.

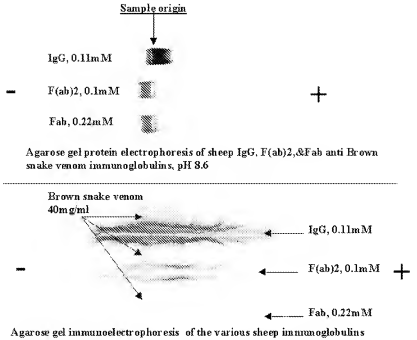


FIGURE 6 Antibrown snake venom immunoglobulins IgG, F(ab)2, Fab (sheep). The whole IgG and $F(ab')_2$ fragment react with the brown snake venom, and there is no visible immunological reaction with the Fab fragment because Fab molecules are not able to cross link the antigen and form an antigen-antibody precipitate.

VENOMS

Pseudonaja textilis, *Pseudonaja nuchalis*, *Pseudonaja affinis*, and *Pseudonaja inframacula* venoms were supplied by Venom Supplies, Pty. Ltd., Tanunda, South Australia.

PAPAIN AND PEPSIN

Papain and pepsin were obtained from Sigma (USA).

Brown Snake Antivenom Preparation

VENOMS

Brown snake venom. Adult male and female snakes, aged greater than 5 years were housed in facilities offering optimum

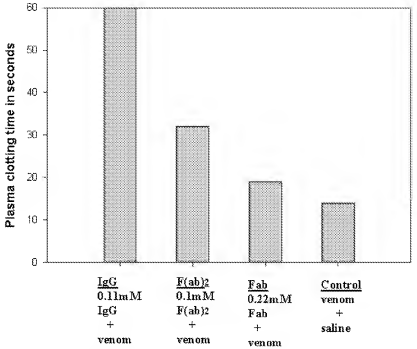


FIGURE 7 Effectiveness of affinity purified antibrown snake venom ovine IgG, F(ab)2, and Fab fragments at neutralizing the procoagulant venom toxin. 100 μL of brown snake venom (1 mg/mL) was mixed with 100 μL of IgG (0.11 mM), 100 μL of $F(ab)_2$ (0.1 mM), 100 μL of Fab (0.22 mM), or 100 μL of saline (control). The venom + immunoglobulin mixtures where incubated at 37°C for 15 minutes (including the venom + saline control). The results show that there is a substantial reduction in the procoagulant neutralization when the IgG immunoglobulin is fragmented to F(ab')2 and Fab. **Note: The immunoglobulin fractions by themselves had neither procoagulant nor anticoagulant action, and in the assay condition described gave the same clotting time as the plasma + saline blanc (~500 sec).**

temperatures (25–30°C), and were fed rodents (specially bred for this purpose). The food was sometimes supplemented with vitamins and the snakes were exposed to the natural photoperiod. The snakes were routinely checked for health status using company protocol and standards. The venom collected from the snakes is pooled and freeze dried.

Immunization protocol. Groups of 20 Brown Pullet hens were primed on Day 0 with 0.5 mL subcutaneous injection of 0.5

LD_{50} of a venom (mixture of South Australian and Queensland *Pseudonaja textilis* venoms). The venom mixture was dissolved in PBS (phosphate buffered saline) and emulsified in 0.5 mL of complete Freund adjuvant. Subsequent doses used a dose of greater than 1.0 LD_{50} and were emulsified in incomplete Freund adjuvant. Adult merino sheep were injected using the methodology outlined above using a total volume of 2 mLs of the antigen/adjuvant mix.

Hens were injected with two subcutaneous injections in the breast with the antigen/adjuvant mix. The injections were repeated every 28 days with steadily increasing doses ranging from 1.0 LD_{50} and higher. Adult merino sheep were injected at four sites proximal to regional lymph nodes.

Separation of water soluble proteins (IgY) from the egg yolk. The egg yolks were separated from the egg white and frozen at –20°C.

One part (volume) of whole frozen egg yolk was thawed in sterile, pyrogen free water (at 4°C) and shaken occasionally. Sodium azide was added to 15 mM concentration. After centrifugation at 5000 rpm for 35 minutes (4°C), the supernatant was separated and chloroform was added (5% v/v final concentration) and the mixture was vigorously shaken to remove any lipid present. This mixture was further centrifuged at 5000 rpm for 35 minutes (4°C) and the supernatant was collected. Sodium chloride was added to a final concentration of 0.15 M and 15 mM sodium azide. This crude egg yolk extract was used as the starting material for the affinity chromatographic separation of the antibrown snake venom immunoglobulins (IgY).

Preparation of sheep serum. The immunized sheep were bled into sterile, pyrogen-free blood collection bags (Advanced Scientifics, Inc.); about 1.0 liter of blood was collected at one bleed. The blood was allowed to clot, the bags were then centrifuged at 4000 rpm (4°C) for 30 min, and the serum separated into sterile, pyrogen-free plastic bottles. To the serum sodium azide was added to 15 mM concentration and 0.2 μ sterile filtered. The serum was used immediately or stored at – 20°C in a nonself-defrosting freezer.

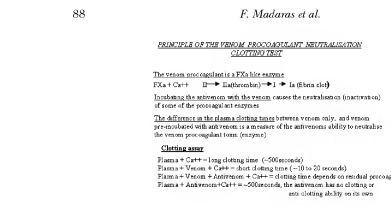
Affinity chromatography. Affinity chromatography was performed using CNBr-activated Sepharose 4B (Pharmacia Biotec). Brown snake venom was immobilized to this matrix (10 mg/mL of gel) using the procedure recommended by the manufacturer. The gel was placed into a chromatography column. The water-soluble protein extract from the egg yolks (or undiluted sheep serum) was applied to this column; the antibrown snake venom immunoglobulins (IgY or sheep IgG) bound to the venom proteins (antigens) insolubilized on the chromatography matrix. When the column was saturated with IgY (or IgG) immunoglobulins, the column was washed with phosphate buffer saline (PBS) pH 7.2. The washing was continued until no protein was detectable in the eluate measured at 280 nm. The buffer was then changed to a 0.1 M glycine/HCl pH 3.1.

The pH in the eluted IgY/IgG fraction was adjusted to 7.2 using 1 M NaOH. The fraction was diafiltered against sterile, pyrogen-free PBS containing 0.22% (w/v) phenol. The diafiltration was done using a 100 K MWCO Vivaflow module (Pyrogen free, Vivascience AG, www. vivascience.com). After the dialysis the protein concentration in the fraction was determined using BCA (Pierce, Protein Assay Kit 23225).

The final antivenom was made by blending the appropriate concentration of IgG (sheep) with the IgY (hen) immunoglobulins. The final antivenom mixture was filtered through a 0.2 μm nonpyrogenic membrane; the antivenom was then tested in various tests as described in "Methods." The antivenom was aliquoted out into pyrogen free glass vials and stored at 4°C.

Fragmentation of IgG to produce F(ab')$_2$ and fab fragments. Affinity purified antibrown snake venom IgG (sheep) was used to prepare F(ab')$_2$ and Fab antivenom immunoglobulin fragments by the method described by Mage (1980). The enzymes, papain, and pepsin (Sigma) were used for the digestion.

Agar gel electrophoresis methodology. The agarose gel protein and immunoelectrophoresis was a modification of the method by Grabar (1957). In brief, 2% (w/v) DNA grade agarose was made up in barbitone buffer pH 8.6. Agar plates (115 x 100 mm and 1.0 mm thick) were cast, 1.4 μL of venom (30 mg/mL) (or other protein IgY, IgG, etc.) was placed into the sample wells, the plates



were placed into an electrophoresis chamber (in a horizontal position) and connected to the cathode and anode chamber buffer by paper wicks. The electrophoresis was performed using 2.0 V cm_2/plate for about 30 min. In these conditions the proteins separate out according to their net electric charge. To visualize the separated proteins, the gel was placed into a solution of 10% (w/v) trichloroacetic acid for 10 min. After this, the gel was placed into a staining solution (Gradipore electrophoresis stain CAT #SG-021) and stained overnight. The gel was distained using a methanol-acetic acid-water solution (1:3:6).

Immunoelectrophoresis. After the agarose protein electrophoresis was completed, the well (a trough cut between the protein, sample-containing gel) was filled with the antibody (antivenom) and left to diffuse into the agar gel at 37°C for 10 hours. The agar gel plate was washed in saline and then dried out. The antibody-antigen precipitin reaction (protein-arcs) were visualized by staining the plate using the process described above.

Methods Used to Test the Brown Snake Antivenom

CLOTTING TESTS

In the tests the antibrown snake antivenoms (ABSA)∗ were used-undiluted and diluted, 1:2 and 1:4. Brown snake venom (South Australia and Queensland) was made up to 1 mg/mL in saline. 0.1 ml of venom was mixed with 0.1 mL of antivenom dilutions and incubated at 37°C for 30 min. As a negative control, 0.1 mL of venom was mixed with 0.1 mL of saline and incubated at 37°C for 30 min.

THE ANTIVEN P/L AND CSL P/L ABSA USED IN THE ASSAY BOTH HAD 1000 UNITS/VIAL POTENCY.

Clotting assay. To 0.1 mL of normal citrated sheep plasma was added 0.1 mL of 0.025 M $CaCl_2$ and 10 μL of the venom antivenom mix, and the clotting time was determined. The assay was done at 37°C.

DETERMINATION OF THE NEUTRALIZATION KINETICS OF THE PROCOAGULANT TOXIN BY THE ANTIVENOM

TEST:. 100 μL of brown snake venom (1mg/mL) was mixed with 100 μL of ABSA (protein concentration 12 mg/mL) and incubated at 37°C. At various time intervals an aliquot was withdrawn and a clotting assay performed.

Clotting assay. To 100 μL of citrated sheep plasma was added 100 μL of 0.025 M $CaCl_2$ at 37°C and to this was added 10 μL of the venom antivenom mix, and the clotting time was determined.

Clotting Assays Using Whole IgG, $F(ab')_2$ and Fab Fragments and Brown Snake Venom

The sheep immunoglobulin fractions, whole IgG, $F(ab')_2$, and Fab were compared for their ability to neutralize the procoagulant in the brown snake venom. The concentrations of the immunoglobulins were adjusted so as to be equivalent (equimolar): IgG, 0.11 mM (16.5 mg/mL); $F(ab')_2$, 0.1 mM (12 mg/mL); Fab, 0.22 mM (11 mg/mL). (*Note! IgG and $F(ab')_2$ have a valency of **2**, while Fab has a valency of **1**.)*

Clotting tests. 100 μL of brown snake venom (1 mg/mL) was mixed with either 100 μL of IgG (0.11 mM), 100μL of $F(ab')_2$ (0.1 mM), 100μL of Fab (0.22 mM), or 100 μL of saline (control). The venom + immunoglobulin mixtures where incubated at 37°C for 15 minutes (including the venom + saline control).

Clotting Assay: This was done by adding to 100 μL of citrated sheep plasma, 100 μL of 0.025 M $CaCl_2$ and 10 μL of the venom and immunoglobulin mix (or 10 μL of the venom + saline control). As soon as the 10 μL of the venom-immunoglobulin mix was added, a stop watch was started and the clotting time of the citrated plasma determined. The clotting assay was done at 37°C.

Mouse protection tests. Mouse protection tests were based on a modified version of LD_{50} experiments described in Broad et al. (1979) and Sevcik (1987). Briefly, the method used was as follows:

Mice, SPF IMVS Balb/c 28±2 gm male, in groups of two, were injected intraperitonealy with serial dilutions of antivenom with 5 LD_{50} doses of the respective venom, which had previously been incubated for 60 minutes at ≤37°C. Antivenom dilutions with BSA used were 1:2, 1:4, 1:8, 1:16, 1:32, and if necessary, 1:64 and 1:128.

Results

Affinity Cchromatography

The eluted fractions were continually monitored at 280 nm for the presence of proteins. (**A** 280 nm). The bound antibrown snake venom immunoglobulins (IgY/IgG) elute as a single peak of proteins.

Brown Snake Venom

Immunoelectrophoresis

Mouse Protection Tests

PSEUDONAJA TEXTILIS

Antivenom potency is expressed as the number of units of antivenom activity in 1 ml of antivenom solution. A Unit of Antivenom Activity is defined as the amount (volume) of antivenom that will neutralize 10 μg of venom.

CSL brown snake antivenom (BSAV) control: 1:32 was the highest dilution that protected the mice.

0.1/32 mL of the antivenom neutralized 10 μg of venom.
0.003125 mL of antivenom neutralized 10 μg of venom.
Thus, 1.0 mL of antivenom was 1/0.003125 = 320 units of antivenom activity.

Antiven Pty Ltd Brown snake (*Pseudonaja* spp.) antivenom: 1:64 was the highest dilution to protect the mice.

0.1/64 mL of antivenom neutralized 10 μg of venom.
0.0015625 mL of antivenom neutralized 10 μg of venom.
Thus 1.0 mL of antivenom has 1/0.0015625 = 640 units of antivenom activity.

Antiven Pty Ltd Brown snake (*Pseudonaja* spp) antivenom had comparable potency of 640 units/mL of antivenom activity against the venoms of *Pseudonaja nuchalis, Pseudonaja affinis,* and *Pseudonaja inframacula.* All of these venoms were tested individually

Clotting Test Results

To 0.1 mL of citrated sheep plasma was added 0.1 mL of 0.025 M $CaCl_2$ and 10 μL of the venom antivenom mix and the clotting time determined. The assay was done at 37°C. Note: neither of the antivenoms (Antiven Pty Ltd or CSL Ltd) had any effect on the plasma clotting time by themselves, and gave the same clotting time as the plasma-saline blanc ~500 seconds ("plasma recalcification time"). Both antivenoms had 1000 U/vial activity.

Discussion

The IgY/IgG Brown snake veterinary antivenom in the various tests so far has proven it self to be an effective and safe immunotherapy in the treatment of brown snake envenomation. The ratio of IgY to IgG in the antivenom is about 1:1.

The rational for using hen IgY as a proportion of the antivenom was to reduce the amount of mammalian IgGs in the whole antivenom. The sheep IgGs were used as whole molecules, and it was felt that the antivenom would be safer and less likely to cause complications due to IgG-Fc mediated complement activation, if a portion of the antivenom contains IgYs, which don't seem to activate mammalian complement (Thalley and Carroll, 1990).

The antivenom is a composite (IgY/IgG) immunoglobulin because the IgYs alone are not able to neutralize all the venom toxins found in the brown snake venom, in particular, the low-molecular-weight (~10 kDa), postsynaptic neurotoxins. Sheep IgGs have the ability to neutralize all the various high and low

molecular weight toxins. In general, we have found that hens produce very effective antibodies against venom toxins whose molecular size is >30 kDa; however, they show very poor immune response to low molecular weight toxins. The brown snake venom contains large amounts of a high molecular weight (>200 kDa) clotting enzyme (see Fig. 2, brown snake venom agar gel and protein scan). This venom toxin is the main cause of death in large animals and humans. The sheep and hen antibodies produced against this enzyme were both able to neutralize it effectively.

When the whole affinity purified sheep IgGs were enzyme digested to yield $F(ab')_2$ and Fab fragments, it was found that the fragments' ability to neutralize the procoagulant venom toxins was substantially reduced compared with the whole IgG molecule (see Figs. 6 and 7). This fact could explain why the CSL Brown snake antivenom shows poor neutralizing ability against the procoagulant toxin. This antivenom is a $F(ab')_2$ (nonaffinity purified) immunoglobulin product. The fact that the (sheep) brown snake antivenom's ability to neutralize the principal venom toxin is substantially impaired when it is reduced to a $F(ab')_2$ and Fab fragment indicates that perhaps safety vs. efficacy benefits of immunoglobulin fragmentation should be re- evaluated. It was reported (Gutierrez et al., 2003) that there could be an advantage in using Fab immunoglobin fragments in neutralizing low-molecular weight toxins; however, they believe that treating large molecular weight components, such as those found in viperid venoms, requires a different approach. No differences were shown (Leon et al., 2001) between IgG and $F(ab')_2$ in neutralizing lethal and defibrinating activities induced by *Bothrops asper* venom, but the allergenic potential of IgG was greater. Fab fragments of immunoglobins were highly effective (Jones and Landon, 1999) compared with CSL Pty Ltd. Brown snake antivenom (which is a $F(ab')_2$ type antivenom) at reversing neurotoxicity in *Pseudonaja* sp. Venoms. However, the authors did not compare their Fab fragment with an $F(ab')_2$ and whole IgG molecules made from the same population of immunoglobulins used to make the Fab fragment. They did not test the efficacy of Fab fragments against the clotting components.

Most antivenom manufacturers these days use $F(ab')_2$ or Fab immunoglobulin fragments in their products. It is possible that

in some instances this leads to reduced efficacy (compared to the whole molecule) and large amounts of antivenom is required to reverse a particular envenomation. This could be the reason for the need to infuse large amounts of foreign proteins into some patients, thus negating any safety benefits that might have been gained by removing the immunoglobulin Fc fragment. We feel that a thorough investigation of this possibility is warranted. It also appears that by digesting the IgG molecule, its ability to cross-link and precipitate the antigen is impaired in $F(ab')_2$ fragments and naturally absent in Fab (see Fig. 6). Cross-linking and precipitating the antigen are the main mechanisms by which IgGs neutralize soluble protein antigens. If enzyme digestion of the IgGs impair this function, then this would further degrade the immunoglobulins' ability to neutralize the antigen. We feel that using affinity purification in antivenom manufacture potentially results in a much safer (fewer proteins) and more effective antivenom, than just making a immunoglobulin fraction by precipitation or simple chromatography. A well-designed affinity chromatography process is cost and time effective and well suited for sterile operating conditions.

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Immunogenicity and kinetics of distribution and elimination of sheep digoxin-specific IgG and Fab fragments in the rabbit and baboon

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(*Accepted for publication* 29 *September* 1978)

SUMMARY

To evaluate the relative merits of purified IgG and Fab preparations of defined specificity for potential clinical use, immunogenicity studies were carried out in baboon and rabbit experimental models. Distribution and elimination kinetics of purified sheep digoxin-specific IgG and Fab fragments were also studied following intravenous administration to baboons. Serial plasma and urine Fab concentrations were determined from trichloroacetic acid-precipitable ^{125}I counts from pre-labelled preparations and also by measurement of the antibody's functional ^{3}H-digoxin binding capacity. Results were compared with data obtained from IgG by ^{3}H-digoxin binding. Kinetic data analysed by computer-fitted functions demonstrated that plasma Fab disappearance was best described by a tri-exponential function, whereas a bi-exponential function best described the IgG data. Initial distribution half-life ($t_{\frac{1}{2}}$) of Fab (0·28–0·32 hr) was considerably shorter than that of IgG (4·0 hr) and contributed a greater proportion of the total fall in plasma level over 24 hr. Fab elimination $t_{\frac{1}{2}}$ (9–13 hr) was also shorter than IgG (61 hr), but appreciably longer than earlier estimates in rabbits, guinea-pigs, rats and mice. The total volume of distribution of Fab was 8·7 times greater than that of IgG measured by the same method. Over the first 24 hr after administration 30–45% of administered Fab was recoverable in active form in urine, while 93% of total administered ^{125}I counts from ^{125}I-Fab preparations (bound and free) could be recovered. Less than 1% of administered IgG binding activity was recovered in urine during the initial 24 hr.

The relative immunogenicities of sheep digoxin-specific IgG and Fab fragments were studied in six baboons. Both IgG and Fab elicited prompt immune responses when injected intramuscularly with Freund's complete adjuvant. Intravenous injection of soluble sheep IgG resulted in a prompt immune response in one baboon while repeated injections caused only a late, weak response in a second animal. Soluble sheep Fab fragments elicited only delayed and weak responses in the two baboons thus challenged. Further immunogenicity studies in nineteen rabbits showed significantly earlier and greater antibody responses to intravenously administered sheep IgG antigen than to Fab fragments derived from the same IgG population. These studies demonstrate that digoxin-specific Fab fragments undergo more rapid and extensive distribution to the extravascular compartment and also more rapid renal excretion than IgG. Furthermore, Fab fragments are significantly less immunogenic than the parent IgG population. These data indicate potentially important therapeutic advantages for digoxin-specific Fab compared with IgG when administered for the reversal of life-threatening digitalis toxicity.

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0099-9104/79/0060-0384 $02.00

INTRODUCTION

The exquisite specificity of the immune response provides a rationale for the use of antibodies in the diagnosis and therapy of a number of human disease states (Haber & Krause, 1977). The clinically important problem of digitalis toxicity has been attacked by the use of cardiac glycoside-specific antibodies (Butler & Chen, 1967; Smith, Butler & Haber, 1977; Smith *et al.*, 1976). Recent experiments have demonstrated the reversal of various pharmacological and toxic effects of cardiac glycosides by specific antibodies (Smith *et al.*, 1977; Schmidt & Butler, 1971; Curd *et al.*, 1971; Smith, 1972; Gold & Smith, 1974). Major problems in the clinical use of heterologous antibodies, however, include the possibility of hypersensitivity reactions to foreign protein, manifest acutely as anaphylaxis, or as a delayed serum sickness reaction. One approach to this problem has been the preparation and purification of digoxin-specific Fab fragments (Curd *et al.*, 1971). Fab fragments provide several theoretical advantages over intact antibody, including absence of complement-binding determinants of the Fc fragment.

Previous studies have demonstrated that Fab fragments are excreted in the urine to a considerably greater extent than IgG (Spiegelberg & Weigle, 1965; Butler *et al.*, 1977). High affinity specific Fab fragments substantially enhance the rate of urinary excretion of digoxin (Butler *et al.*, 1977) and digitoxin (Ochs & Smith, 1977) compared to IgG. The present experiments were undertaken to test the hypothesis that Fab fragments, having a molecular weight of 50,000, are more rapidly and extensively distributed in the body after intravenous administration than the parent IgG molecule. In addition, we have characterized the immunogenicity of purified ovine digoxin-specific IgG and Fab fragments in both rabbits and baboons to test the hypothesis that the smaller and more rapidly excreted Fab fragment, lacking the antigenic determinants of the Fc fragment, is less immunogenic.

MATERIALS AND METHODS

Preparation and purification of sheep digoxin-specific IgG and Fab fragments. Sheep were immunized with a digoxin-human serum albumin conjugate, boosted, and bled as described previously (Curd *et al.*, 1971). Antisera were characterized in terms of the quantity and affinity of digoxin-specific antibody (Smith, Butler & Haber, 1970). Single bleedings with average intrinsic affinity constants for digoxin ranging from $7{\cdot}0\times10^{9}$ to $1{\cdot}5\times10^{10}\,\text{M}^{-1}$ were pooled and purification of digoxin-specific IgG and Fab fragments was carried out as described previously (Smith *et al.*, 1976; Curd *et al.*, 1971) using an affinity chromatographic technique. After purification, Fab preparations were chromatographed on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, New Jersey) to remove trace amounts of incompletely digested IgG. Preparations were stored frozen in phosphate buffered saline (0·15 M NaCl, 0·01 M NaH_2PO_4 adjusted to pH 7·4) at −20°C at a concentration of 5·0 mg/ml. Immediately prior to immunization procedures, IgG and Fab preparations were thawed, centrifuged at 29,000 *g* for 1 hr, and passed through a 0·22 μm Millipore filter. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of aliquots of these preparations was carried out according to the method of Weber & Osborn (1969), omitting the 2-mercaptoethanol step, and confirmed the absence of detectable protein bands other than those corresponding to a molecular weight of 150,000 in the case of IgG, or 50,000 for the Fab preparation.

Radioiodination of sheep IgG and Fab preparations. Aliquots of purified digoxin-specific sheep IgG and Fab fragments were labelled with ^{125}I for use in studies of kinetics of distribution and elimination, and of immunogenicity. The lactoperoxidase method of Marchalonis (1969) was used. After radioiodination, the reaction mixtures were passed over a 1 × 10 cm column of Sephadex G-75 over which 10 mg of unlabelled Fab had previously been passed. The first ^{125}I-containing material eluted from this column was collected, dialysed against three changes of two litres of phosphate buffered saline at 4°C, then stored in aliquots at −20°C. Identical mobility of radiolabelled and unlabelled protein was demonstrated by polyacrylamide gel electrophoresis and gamma scintillation counting of serial 2·0 mm gel sections (Hopkins, Wagner & Smith, 1976). Greater than 98% of initial ^{125}I counts in Fab and IgG preparations were precipitable in the presence of serum or added serum albumin following the addition of an equal volume of 12% trichloroacetic acid (TCA), cooling in crushed ice and centrifugation at 5000 *g* for 20 min.

Fab and IgG distribution and elimination kinetics. Digoxin-specific IgG and Fab concentrations in kinetic studies in baboons were determined by the measurement of ^{3}H-digoxin binding capacity of plasma or urine samples at saturation. In addition, the measurement of total and TCA-precipitable ^{125}I tracer counts from ^{125}I-labelled Fab fragments was used for studies of Fab kinetics.

Assay of IgG and Fab by ^{3}H-digoxin binding capacity. Assay of plasma and urine for Fab and IgG digoxin binding capacity was carried out by a modification of a charcoal method previously developed for the rapid separation of antibody-bound and free cardiac glycoside (Smith & Skubitz, 1975; Skubitz, O'Hara & Smith, 1977). Maximal binding of ^{3}H-digoxin by Fab or IgG was attained in the presence of excess ^{3}H-digoxin. Residual unbound or free ^{3}H-digoxin was then removed by brief exposure to dextran-coated charcoal. Fab or IgG concentrations were determined from the amount of ^{3}H-digoxin bound.

The assay system consisted of a 500 μl vol. of sample plasma, suitably diluted with pre-treatment plasma, incubated in 700 μl phosphate buffer containing 5, 25 or 50 ng of ^{3}H-digoxin, sp. act. 1·1 μCi/nmol (New England Nuclear Corp., Boston, Massachusetts). After 30 min, 1·0 ml was added to and thoroughly mixed with 0·5 ml of dextran-coated charcoal suspension (Herbert *et al.*, 1965) contained in a 6·0 ml disposable plastic syringe, the outlet from which was plugged with a small amount of clay ('Seal-Ease', Clay-Adams, Parsipanny, New Jersey). After 30 sec, the syringe plunger was used to force the suspension rapidly through a 1·0 μm Millipore filter (Millipore Corp., Bedford, Massachusetts) contained in a 25 mm diameter Swinnex adapter (Millipore Corp.). Preliminary studies showed 30 sec to be an adequate time for charcoal binding of free and albumin-bound ^{3}H-digoxin to occur without a significant alteration in antibody-bound ^{3}H-digoxin. For assay of antibody-binding activity in urine, urine plus buffer in a final volume of 700 μl were incubated with excess ^{3}H-digoxin and then further treated as described above.

1·0 ml of filtrate was added to 15 ml of liquid scintillant (Instagel, Packard Instrument Corp., Downers Grove, Illinois) and counted in a liquid scintillation spectrometer (Packard Model 3320). Filtrate counts were corrected for background and small contributions from free hapten and non-hapten counts not bound by charcoal as described previously (Skubitz & Smith, 1975). In each assay, sample sizes were chosen such that at least two of the three hapten quantities added (5, 25 and 50 ng) were sufficient to provide at least a two- to three-fold excess of hapten over antibody-binding sites, resulting in the saturation of ^{3}H-digoxin binding with at least two of the three hapten concentrations. IgG and Fab concentrations were calculated from the known specific activity of bound ^{3}H-digoxin, assuming molecular weights of 150,000 and 50,000 for IgG and Fab, respectively.

Protocol for kinetic studies. A mixture of digoxin-specific ^{125}I-Fab and unlabelled Fab was administered on two separate occasions at least 1 week apart in three conscious healthy female baboons (*Papio anubis*, 11 to 16 kg) with normal renal function (plasma creatinine concentration range 0·9–1·3 mg/dl). Serial plasma and urinary Fab concentrations were determined from total and TCA-precipitable ^{125}I counts and by ^{3}H-digoxin binding as a measure of Fab functional capacity. In a final study in each animal, digoxin-specific IgG was administered and serial IgG concentrations determined from ^{3}H-digoxin binding capacity.

Following intramuscular ketamine HCl (Bristol Labs, Syracuse, New York) anaesthesia (100 mg), the femoral vein was isolated in a sterile fashion and cannulated for subsequent venous sampling. A scalp vein needle was placed in a fore-limb vein for antibody administration. Urine was collected via a catheter passed per urethra and left indwelling in the bladder. The baboon was placed in a restraining chair and allowed to awaken. Fab fragments were administered intravenously in a dose of 5 mg/kg containing 4–6 μCi ^{125}I. IgG was administered also in a dose of 5 mg/kg body weight. Infusions were given over 2–4 min via the scalp vein needle, which was then removed. Blood samples were collected prior to antibody administration, upon completion of infusion (zero time) and 5, 10, 15, 30, 45 and 60 min and 1·5, 2·0, 2·5, 3·0, 4·0, 6·0, 8·0, 12·0, 24·0 and 48·0 hr later. In animals given IgG, further blood samples were collected every other day up to day 14. 24 hr after administration cannulae were removed and the animal was returned to a cage. Subsequent blood samples were collected by venipuncture following intramuscular ketamine HCl (100 mg) administration. Immediately after blood collection, plasma was separated and 1·0 ml aliquots were used for measurement of total and TCA-precipitable ^{125}I counts. Further aliquots were frozen and stored at −70°C. Urine was collected at 0–2, 2–4, 4–6, 6–12 and 12–24 hr after IgG or Fab administration and treated as described for plasma samples. Plasma and urine samples containing ^{125}I-Fab were counted in a gamma scintillation counter (Searle Analytic Inc.).

Kinetic analysis. Plasma Fab and IgG concentrations following intravenous infusion were analysed by computer using iterative non-linear least squares regression techniques (Loo & Riegelman, 1970; Greenblatt & Koch-Weser, 1975). Data points from individual animals were fitted to the following two functions: $C = Ae^{-\alpha t} + Be^{-\beta t}$ (equation 1); and $C = Ae^{-\alpha t} + Be^{-\pi t} + Be^{-\beta t}$ (equation 2), where C is the plasma Fab or IgG concentration at time t after the end of the infusion, A, P, and B are 'hybrid' intercept terms, and α, π and β are 'hybrid' exponents. Coefficients were corrected for the infusion period (Loo & Riegelman, 1970). For each animal the choice between the suitability of equations 1 and 2 was made by comparing the sum of squares of weighted residual errors and by assessing the random nature of scatter of the actual data points about the fitted function. The appropriate function was then used to calculate the following kinetic variables (Greenblatt & Koch-Weser, 1975; Wagner, 1975): distribution half-life ($t_{\frac{1}{2}\alpha}$), intermediate 'pi' half-life ($t_{\frac{1}{2}\pi}$) when appropriate, elimination half-life ($t_{\frac{1}{2}\beta}$), volume of the central compartment (V_1), total apparent volume of distribution using the 'area' method (V_d), and total clearance.

Group means were compared using the Student's *t*-test and paired Fab concentrations measured from ^{125}I counts and ^{3}H-digoxin binding were analysed using the paired *t*-test (Snedecor & Cochran, 1967).

Immunization procedures. Baboons and rabbits were immunized both by depot injections of antigens in Freund's complete adjuvant or by repeated intravenous injections of soluble antigen.

Baboon immunizations. Six healthy adult baboons housed at the New England Regional Primate Research Center were studied. 50 ml of control serum was obtained from each animal prior to immunization. One animal received a single challenge of 20 mg of purified sheep digoxin-specific IgG as four 1·0 ml intramuscular injections of 5·0 mg each in Freund's complete adjuvant. A second animal was similarly challenged with 20 mg of purified sheep digoxin-specific Fab fragments. The remaining four baboons received serial intravenous injections of 1·0 mg/kg of specific IgG (ultracentrifuged and filtered as noted above) at 0, 2, 4, 6, 8, 10, and 12 weeks (two animals), or of specific Fab fragments according to the same dosage and time schedule (two animals). Bleedings from an antecubital vein were obtained, after ketamine sedation, just prior to antigen administration and at 2, 4, 6, 8, 10, 12 and 14 weeks.

Rabbit immunizations. Nineteen healthy adult New Zealand white rabbits were immunized with purified sheep digoxin-specific IgG or Fab fragments. Pre-immune bleedings were obtained from all animals. One animal received footpad injections of 1·0 mg/kg of IgG in Freund's complete adjuvant and two received Fab by the same method, route, and dose. Eight rabbits received initial intravenous injections of 1·0 mg/kg ultracentrifuged and filtered IgG followed by identical booster injections at 3 and 6 weeks. Eight rabbits were given repeated 1·0 mg/kg intravenous Fab injections according to the same schedule.

Two additional rabbits were immunized with multiple toe-pad injections of baboon IgG, boosted, and bled after 6 weeks to obtain rabbit antibaboon IgG for use in the assay system described below.

Quantification of the immune response to sheep IgG and Fab. Antibody formation by baboons and rabbits was detected and quantified by radioiodine labelling of antigen, incubation of labelled antigen with serum from immunized baboons or rabbits, and then incubation with a second antibody against baboon or rabbit IgG to assure precipitation of soluble immune complexes.

Sera from rabbits challenged with sheep digoxin-specific IgG or Fab fragments were assayed by initial incubation of 0·1 ml of antiserum with at least 10^5 ct/min of ^{125}I-labelled antigen in a total volume of 1·0 ml of phosphate buffered saline for 1 hr at 37°C, followed by incubation for an additional 23 hr at 4°C. Goat anti-rabbit IgG (Hyland) was then added, the amount of which had been determined previously in precipitin experiments to give a maximum yield of precipitated rabbit IgG. Incubation was again carried out at 37°C for 1 hr and at 4°C for 23 hr. Precipitates were centrifuged at 5000 *g* for 20 min and the supernatant carefully decanted and saved. Pellets were washed twice with 1·0 ml cold phosphate buffered saline and counted in a gamma well scintillation counter. Counts present in the initial supernatant and washes were also measured and total counts recovered for each sample determined. This recovery value was at least 95% of total antigen counts in the system in each instance. Residual ^{125}I counts in the final washed pellet for samples of pre-immune rabbit serum samples did not exceed 2% of antigen counts added. In contrast, with antiserum containing specific antibody in excess of the amount of ^{125}I-labelled antigen in the system, at least 90% of total antigen counts were recovered in the final washed precipitate. For each set of unknown antisera from intravenously challenged animals, ^{125}I-labelled antigen counts added were at least two-fold greater than the counts present in the final washed precipitate.

The immune responses in baboons were estimated by a similar method in which rabbit antibody against baboon IgG was used to form the final triple precipitate. Validation of the method was carried out as described above for rabbit immune responses, and results were expressed as a percentage of the total ^{125}I-antigen counts present in the final washed precipitate.

Further calibration experiments were carried out to permit an approximation of absolute concentrations of antibody formed by rabbits against sheep IgG or Fab. For this purpose, single bleedings at the height of the immune response were selected from rabbits challenged with multiple toe-pad injections of sheep IgG or Fab in Freund's complete adjuvant. Serial dilutions of serum from these bleedings were incubated with the appropriate ^{125}I-labelled antigen as described above, and ^{125}I counts and total protein content (Lowry *et al.*, 1951) of washed precipitates were determined. At the peak of the precipitin curve, precipitate counts present in the responder serum used as standard were at least fifty times greater than values for pre-immune serum from the same animal. Using the known protein content of the sheep IgG or Fab antigen relative to the ^{125}I counts present, the protein content of the washed pellet contributed by specific rabbit anti-sheep IgG or rabbit anti-sheep Fab was determined. At the peak of the precipitin curve, at least two dilutions of rabbit antiserum gave antibody protein estimates that agreed within 10%. Serial dilutions of these standard antisera were then made, and run with each set of unknown antisera as a standard curve. Antibody concentrations in unknown samples were estimated from this standard curve, bearing in mind that the antigen–antibody combining ratio of the test antiserum could differ from that of the standard antiserum. The amount of antibody present in the standard antiserum was well in excess of the response of any of the intravenously challenged animals, so that unknown values were always determined by interpolation between two known antibody concentrations. The sensitivity of the method used allowed the measurement of 0·05 mg/ml antibody concentrations; agreement between duplicate samples was 15% or better at 0·05 mg/ml and 10% or better above 0·1 mg/ml.

RESULTS

Kinetics of sheep Fab fragments in baboons

Fab concentrations at the completion of intravenous infusion (zero time) measured from TCA-precipitable ^{125}I counts (161·6±12·9 s.e. μg/ml) and from ^{3}H-digoxin binding capacity (141·0±11·5 μg/ml) were similar when the paired data in individual animals (paired *t*-test) or the group means (Student's *t*-test) were compared. The time course of the fall in mean plasma Fab concentration, expressed as a percentage of zero time values, is illustrated in Fig. 1. Plasma Fab levels fell rapidly from the time when infusion was completed. Mean percentages of zero time plasma Fab concentrations for all animals analysed by TCA-precipitable ^{125}I counts were somewhat higher than corresponding values determined by ^{3}H-digoxin binding capacity for times from 6–48 hr, but values overlapped and the means did not differ significantly. Values at earlier times determined by ^{3}H-digoxin binding capacity were significantly less.

TABLE 1. Kinetic parameters determined from two methods of plasma Fab estimation after intravenous Fab administration in baboons

	^{3}H-digoxin binding method						TCA-precipitable ^{125}I method					
Animal	$t_{\frac{1}{2}}\alpha$ (hr)	$t_{\frac{1}{2}}\pi$ (hr)	$t_{\frac{1}{2}}\beta$ (hr)	V_1 (ml/kg)	V_d (ml/kg)	Cl (ml/kg/min)	$t_{\frac{1}{2}}\alpha$ (hr)	$t_{\frac{1}{2}}\pi$ (hr)	$t_{\frac{1}{2}}\beta$ (hr)	V_1 (ml/kg)	V_d (ml/kg)	Cl (ml/kg/min)
1. a	0·30	2·57	—*	50·5	621	—*	0·06	0·08	13·9	25·7	300	0·25
b	0·34	2·48	—*	35·9	483	—*	0·46	1·4	11·2	29·5	270	0·28
2. a	0·18	0·63	7·04	35·7	566	0·93	0·14	0·95	21·7	29·4	400	0·21
b	0·34	1·28	9·4	29·5	568	0·70	0·80	0·70	10·7	37·7	230	0·25
3. a	0·18	1·17	12·0	13·2	308	0·29	0·37	1·19	12·6	20·6	260	0·23
b	0·34	0·64	8·0	14·8	218	0·32	0·13	0·77	11·6	26·0	218	0·30
Mean	0·28	1·46	9·1	29·9	461	0·56	0·33	0·85	13·6	28·2	280	0·25
s.e.m.	0·03	0·35	1·08	5·2	66	0·15	0·11	0·19	1·7	2·3	27	0·01

* Late data points not available.
$t_{\frac{1}{2}}\alpha$ = Distribution half life.
$t_{\frac{1}{2}}\pi$ = Intermediate half life.
$t_{\frac{1}{2}}\beta$ = Elimination half life.
V_1 = Volume of central compartment.
V_d = Total volume of distribution (area method).
Cl = Total clearance.

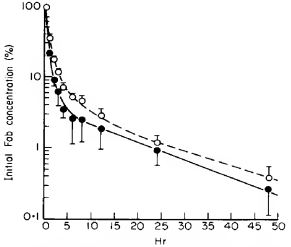

FIG. 1. Time course of fall in plasma concentrations of heterologous Fab fragments. Results of six experiments in three baboons are shown. Purified sheep digoxin-specific Fab fragments were given intravenously. Plasma concentrations as a percentage of the value measured at completion of administration are plotted against time. (○– – –○) Data obtained from ^{125}I-labelling experiments; (●———●) mean values determined by ^{3}H-digoxin binding capacity. Vertical bars denote 1 s.e.

Table 1 shows the kinetic values derived from computer-generated functions which best fit the data from each method of plasma Fab estimation. Plasma Fab fragment clearance curves, determined from both assay methods, were best fitted by a tri-exponential function. Alpha or distribution phase half-life determined from ^{125}I counts (0·32 hr) and from ^{3}H-digoxin binding (0·28 hr) were similar. The distribution phase accounted for 63% of the fall in ^{125}I counts and for 76% of the fall in Fab estimated from ^{3}H-digoxin binding. Beta or elimination $t_{\frac{1}{2}}$ was similar when assayed either from TCA-precipitable ^{125}I counts (13·6 hr) or ^{3}H-digoxin binding (9·1 hr) (Table 1). Intermediate or π half lives determined from each method of Fab estimation were also similar (Table 1).

For Fab, the volume of the central compartment measured 28 ml/kg and 30 ml/kg by ^{125}I and ^{3}H-digoxin binding assays, respectively. The total apparent volume of distribution (area method) of Fab estimated from TCA-precipitable ^{125}I counts (280 ml/kg) was less than that estimated by ^{3}H-digoxin binding (461 ml/kg), but both estimates were considerably greater than that of IgG (Table 2). Mean Fab clearances determined from TCA-precipitable ^{125}I counts and from ^{3}H-digoxin binding were 0·25 and 0·56 ml/kg/min, respectively (Table 1).

Total recovery of ^{125}I (TCA-precipitable and supernatant) in urine excreted during the initial 24 hr after Fab administration averaged 93%. In the same period, TCA-precipitable ^{125}I was considerably less, representing 46% of the administered dose. Recovery of Fab measured from ^{3}H-digoxin binding was 30%. Recovery values for total ^{125}I, TCA-precipitable ^{125}I and Fab determined from ^{3}H-digoxin binding for each time period during the initial 24 hr following administration are shown in Fig. 2. It is of

TABLE 2. Kinetic parameters determined from ^{3}H-digoxin binding after intravenous administration of digoxin specific IgG in baboons

Animal	$t_{\frac{1}{2}}\alpha$ (hr)	$t_{\frac{1}{2}}\beta$ (hr)	V_1 (ml/kg)	V_d (ml/kg)	Cl (ml/kg/min)
1.	8·25	70·8	32·7	65·9	0·011
2.	1·15	35·2	15·3	23·5	0·007
3.	2·49	77·1	38·7	70·0	0·010
Mean	3·96	61·0	28·9	53·1	0·009
s.e.	2·20	13·0	7·0	14·9	0·001

TABLE 3. Antibody responses in rabbits given purified sheep digoxin-specific IgG or Fab intravenously*

		1	2	3	4	5	6	7	8	9	10	11
IgG												
Rabbit	752	0	0·1	0	0·2	(Died)						
	753	0	0·6	0·5	3·3	3·1	2·9	2·8	3·0	3·1	2·0	1·5
	754	0	0	0	0	0	0	0	0	0·5	1·4	1·3
	758	0·6	0·9	0·7	2·6	2·7	2·3	2·7	2·4	2·6	2·0	1·8
	759	0	0	0	0	0	0	0	0	0	0	0
	760	0	0·1	0·3	0·1	0·8	0·5	1·2	1·9	1·8	1·0	0·5
	764	0	0·1	0·3	2·7	1·8	1·8	2·6	2·4	2·3	1·5	0·7
	765	0	0·3	0·3	0·4	0·2	0·2	1·0	0·9	0·8	1·2	1·4
Mean		0·075	0·26	0·26	1·16	1·23	1·10	1·47	1·51	1·59	1·30	1·03
s.e.		0·075	0·115	0·091	0·506	0·494	0·457	0·467	0·460	0·442	0·259	0·243
Fab												
Rabbit	750	0	0	0	0·1	0	0	3·0	3·4	3·2	1·8	1·0
	751	0	0	0	0	0	0	0·5	0·3	0·2	0·1	0
	755	0	0	0·1	0·6	0·1	0·3	2·0	1·6	1·6	1·0	0·8
	756	0	0	0	0·3	0	0·1	0·7	0·6	0·5	0·2	0·1
	757	0	0	0	0	0	0	0·8	0·7	0·5	0·5	0·4
	761	0	0	0·1	0·4	1·0	0·6	1·7	1·5	1·4	0·8	0·6
	762	0	0	0	0	0	0·1	0·5	0·3	0·3	0·1	0
	763	0	0	0	0	0	0	0	0	0	0	0
Mean		0	0	0·025	0·175	0·138	0·138	1·15	1·05	0·963	0·563	0·362
s.e.		0	0	0·016	0·082	0·124	0·075	0·352	0·391	0·377	0·218	0·141

* 1·0 mg/kg body weight IgG or Fab injected intravenously at 0, 3, and 6 weeks. Responses are expressed in mg of specific antibody per ml plasma.

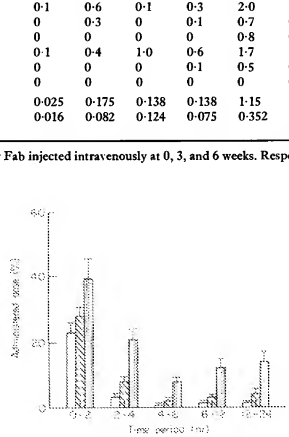

FIG. 2. Urinary recoveries of ^{125}I-Fab and ^{3}H-digoxin binding activity. Results of six experiments in three baboons are summarized. The percentage of total administered dose recovered in urine during five time periods are shown. (▨) Total ^{125}I recovered; (▩) protein-bound ^{125}I defined as TCA-precipitable radioactivity; (□) ^{3}H-digoxin binding capacity recovered. Vertical bars denote 1 s.e.

interest that the proportion of total ^{125}I excreted in TCA-precipitable form declined steadily from a mean of 72% during the first 2 hr to 28% during 12–24 hr, consistent with appreciable catabolism of the ^{125}I-labelled Fab fragment population. Further evidence for catabolism is apparent in the observation that the proportion of TCA-precipitable ^{125}I activity present in urine that could be detected as functionally intact ^{3}H-digoxin binding capacity fell progressively from a mean of 81% for the 0–2 hr collection to

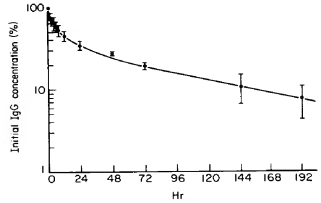

FIG. 3. Time course of fall in plasma concentrations of heterologous IgG. Results of studies in three baboons are shown. Purified sheep digoxin-specific IgG was given intravenously. Plasma concentration values determined from ^{3}H-digoxin binding studies are plotted on the vertical axis as the percentage of binding capacity present at the completion of infusion. Vertical bars show 1 s.e. above and below the mean.

27% for 12–24 hr. Urinary excretion of functional Fab occurred predominantly in the first 2 hr after administration and fell progressively with time.

Kinetics of sheep IgG in baboons

Fig. 3. summarizes the fall with time during the first 24 hr of the mean plasma IgG level expressed as a percentage of the zero time level. In contrast to plasma Fab kinetics, these data show a substantially less rapid fall in plasma IgG concentrations both in the distribution (α) and elimination (β) phases. In Table 2 are shown the kinetic values derived from computer fitting of the time course of plasma IgG concentration. The data were best described by a biexponential function. Compared with Fab, mean IgG distribution (4 hr) and elimination (61 hr) half-lives were significantly greater ($P<0{\cdot}01$). The relatively large scatter in values for individual animals, also noted in a previous study (Butler *et al.*, 1977), raises the possibility that some degree of antibody formation to the prior Fab injection may have occurred. This issue was not further evaluated. In any case, formation of antibody resulting from previous Fab administration would presumably have hastened clearance of IgG, and the substantial differences between Fab and IgG observed could only be underestimates of the true differences if the initial Fab injection did elicit an immune response in any of these animals. The distribution phase accounted for considerably less of the fall in plasma IgG (44%) than with Fab (62–76%). The volume of the central compartment measured after IgG administration (29 ml/kg) was essentially identical to that measure with Fab. The apparent volume of distribution of IgG was 53 ml/kg, approximately twice the measured volume of the central compartment and only about 12% of the volume of distribution of Fab (Table 1). IgG clearance was $0{\cdot}009 \pm 0{\cdot}001$ ml/min/kg over the time period studied, or about 2–4% of Fab clearance.

Recovery of digoxin-specific IgG in baboon urine as judged by ^{3}H-digoxin binding capacity was minimal, and amounted to less than 1% of the administered dose for the period from 0 to 24 hr.

Immunogenicity of sheep IgG and Fab in baboons

Because of the very limited data available on relative immunogenicity of heterologous IgG and Fab in primate species, studies were carried out in six baboons. As shown in Fig. 4, intramuscular injection of sheep IgG or Fab in Freund's complete adjuvant resulted in a prompt antibody response. Bleedings taken 2 weeks after challenge showed a greater response in the animal given IgG, but comparable responses to IgG and Fab were observed thereafter, peaking at 6 weeks and remaining at similar plateau values up to 12 weeks.

One of the two animals challenged intravenously with sheep IgG responded promptly with a level of antibody production at 2 weeks comparable to that of the baboon given intramuscular antigen in Freund's complete adjuvant. The other animal given sheep IgG intravenously responded very differently, failing

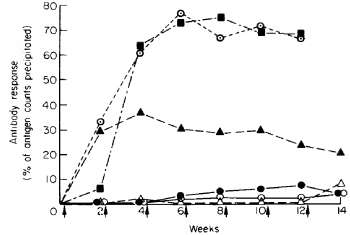

FIG. 4. Antibody responses to purified sheep digoxin-specific IgG and Fab fragments in six baboons. Results from each animal are plotted separately. (○- - -○) Response of an animal given 20 mg of sheep digoxin-specific IgG intramuscularly in Freund's complete adjuvant at zero time; (■- - -■) response of another animal to an intramuscular challenge with 20 mg of purified sheep digoxin-specific Fab fragments in Freund's complete adjuvant. Intravenously challenged baboons received 1·0 mg/kg body weight of antigen as IgG (△, ▲) or as Fab (○, ●) at the times indicated by arrows on the horizontal axis. See Results section for details.

to produce a demonstrable immune response even after six injections at 2-week intervals. At 14 weeks after the seventh injection, a small response finally occurred.

Neither baboon given Fab fragments intravenously showed a detectable response at 2 or 4 weeks. At 6 weeks (2 weeks after the third injection), a small but detectable response occurred in both animals, and both responded to additional challenges with small further increases in anti-sheep Fab levels, which then remained essentially constant from weeks 8 to 14.

Immunogenicity of sheep IgG and Fab in the rabbit

More detailed quantitative immunogenicity studies were carried out in the rabbit. As described in the Materials and Methods, antibody responses were detected by allowing the formation of complexes of antibody with labelled antigen, followed by precipitation with goat antibody against rabbit IgG. Quantification was achieved by comparison with standard curves constructed from data obtained with known amounts of rabbit anti-sheep IgG or anti-sheep Fab, bearing in mind that the estimates given represent

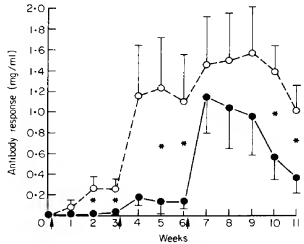

FIG. 5. Antibody responses to purified sheep digoxin-specific IgG and Fab fragments in sixteen rabbits. Data from Table 3 are plotted, with arrows denoting times at which 1·0 mg/kg body weight of ultracentrifuged and filtered IgG (○- - -○) or Fab (●———●) were given intravenously. Vertical bars denote 1 s.e. Asterisks indicate times at which differences in mean responses achieved statistical significance ($P < 0{\cdot}05$).

approximations since the antigen–antibody combining ratio of the test antiserum could differ from that of the standard antiserum.

Three rabbits initially challenged with toe-pad injections of antigen in Freund's complete adjuvant (two Fab, one IgG) showed prompt and vigorous immune responses with serum levels of specific antibody varying from 3·5 to 5·7 mg/ml at 6 weeks. Data from animals challenged with repeated intravenous injections of 1·0 mg/kg of soluble antigen are shown in Table 3 and in summary form in Fig. 5. One (No. 758) of the eight rabbits given sheep IgG responded promptly to the initial challenge with an antibody level of 0·6 mg/ml at 1 week. By the end of 2 weeks, six of the eight animals showed a demonstrable response. A single rabbit (No. 759) did not respond even after three injections and 11 weeks, while a second animal responded only at 9 weeks, well after the third injection. Appreciable responses to the second IgG injection were noted in most animals, resulting in mean specific antibody levels of 1·2 mg/ml at 4 and 5 weeks. After the third injection further increases occurred with the mean response reaching a maximum of 1·6 mg/ml at 9 weeks.

In contrast to the responses observed to heterologous IgG, none of the eight rabbits given sheep Fab showed a demonstrable antibody response at either 1 or 2 weeks, and only two of the eight showed a slight (0·1 mg/ml) response after 3 weeks. The second Fab injection produced measurable antibody responses in four of the eight rabbits, while one animal (No. 763) failed to respond even after the third injection. After the third Fab injection, seven of the eight rabbits produced appreciable amounts of anti-sheep Fab antibody; mean serum concentrations at 7, 8, and 9 weeks were not significantly different from animals receiving IgG. Mean antibody responses to Fab were significantly less ($P<0·05$) than those to IgG at 2, 3, 5, 6, 10 and 11 weeks. Thus, the response of rabbits to sheep Fab were significantly later and of lesser magnitude than responses to the intact parent IgG population.

DISCUSSION

Little attention has been directed toward heterologous Fab fragments as therapeutic agents in immune deficiency states, presumably because their clearance from the body has generally been considered too rapid to be clinically useful. Relatively rapid clearance of Fab fragments can be used to advantage when the objective is rapid neutralization and clearance of a toxic substance, and purified sheep digoxin-specific Fab fragments have been utilized clinically for the reversal of advanced digoxin intoxication (Smith *et al.*, 1976). This therapeutic approach is based on similar binding properties (Skubitz *et al.*, 1977) and the postulated lesser immunogenicity (Butler *et al.*, 1977) of Fab compared with IgG. For urgent clinical situations such as life-threatening digitalis-toxic cardiac arrhythmias, the present study indicates that Fab has another important advantage—more rapid and extensive distribution to its presumed site of action in the interstitial space. Our data also confirm prior studies (Spiegelberg & Weigle, 1965; Butler *et al.*, 1977) indicating substantially more rapid excretion of Fab compared with IgG.

The present studies compare Fab kinetic data based on functional hapten-binding activity with data from radioiodination of the species to be quantified. Although the general similarity of results obtained by the two methods is reassuring, there are sufficient differences (especially in urinary recovery data) to warrant care in the interpretation of data based on radioiodine recovery alone. The present data, based on the lactoperoxidase radioiodination method of Marchalonis (1969), cannot be assumed to be representative of results obtained by other iodination methods.

While metabolism and excretion of exogenously administered homologous and heterologous IgG have been studied extensively in small animals, subhuman primates and man (Janeway *et al.*, 1967; Waldmann & Strober, 1969; Lance, Medawar, & Taub, 1973), relatively few such data are available for Fab fragments. Spiegelberg & Weigle (1965) found the half-life of heterologous Fab in rabbits, guinea-pigs and mice to be less than 12 hr, and Wochner, Strober & Waldmann (1967) reported a half-life of 3·6 hr in mice. Homologous Fab fragments were reported to have a half-life of 1·7 hr in rats (Arend & Silverblatt, 1975). Our estimates, based on computer analysis of longer and more frequent sampling intervals in a large primate species, indicate a longer elimination phase half-life of 9–12 hr. These differences reflect, at least in part, a difference between measurement of total half-life and beta-phase elimination half-life.

The latter measurement, used in the present study, describes elimination from the central compartment after distribution has occurred and is of particular importance in describing the kinetics of elimination of a substance from the body (Fingl & Woodbury, 1975). Other factors contributing to the differences observed include donor and recipient species. The frequency and duration of sampling intervals and the extent of manipulation of the antibody during its preparation. Our data on IgG and Fab kinetics in the baboon correlate well with the half times of about 70 and 15 hr for heterologous IgG and Fab, respectively, recently observed by Yasmeen *et al.* (1976) in the rabbit.

Janeway *et al.* (1968) estimated a half-time of 5 hr in two normal humans for homologous immunoglobulin fragments from plasmin digests, presumed to be similar to Fab fragments. Gitlin *et al.* (1964) found a half-life of 0·3 days in pregnant women for homologous Fab fragments radioiodinated by a technique using nitrous acid. The methods used by Janeway *et al.* (1968) and by Gitlin *et al.* (1964) are sufficiently different from those employed in the present studies to render comparison difficult. As previously observed (Waldmann & Strober, 1969), IgG half-life in the present study was long, and considerably longer than estimates for Fab.

With Fab fragments, the initial or distribution phase of the decline in plasma concentration was not only more rapid but contributed a greater proportion of the total fall in plasma levels than was the case with IgG. Former studies with heterologous Fab fragments have generally not quantified the initial distribution phase following intravenous administration. Arend & Silverblatt (1975), however, observed in rats that homologous Fab fragments underwent more rapid distribution than IgG. Excluding the renal excretion component by nephrectomy, these workers showed that Fab distribution remained more rapid than IgG. On the basis of our data, we conclude that the short Fab distribution half-time and greater total volume of distribution indicate a relatively rapid egress of Fab from the vascular to the interstitial space. Such a result would be predicted on the basis of a comparison of size and molecular weight of the 150,000 dalton IgG and 50,000 dalton Fab molecules.

The volume of distribution of Fab fragments estimated from TCA-precipitable ^{125}I counts was somewhat less than that measured by assay of the antibody's functional capacity. The latter assay resulted in slightly lower plasma Fab levels in the initial hours after Fab administration which would necessarily have influenced V_d, derived from the area under the curve in the present study. Why such a difference in plasma levels should exist is not clear, but it may, in part, result from the retention and non-specific plasma protein binding of free ^{125}I following ^{125}I-Fab catabolism or degradation. The possibility of co-precipitation of a fraction of free ^{125}I counts cannot be excluded. The important point, however, is that the Fab distribution volume estimated by both methods is substantially greater than plasma volume, and when estimated by ^{3}H-digoxin binding it is nearly nine times the distribution space of IgG.

Following ^{125}I-Fab administration, 93% of the administered ^{125}I was recoverable in urine, but only 30–46% was accounted for by intact Fab as indicated by TCA precipitation and ^{3}H-digoxin binding experiments. These results confirm and extend those found in small animals. Arend & Silverblatt (1975) found that 90% of injected counts were recoverable in the urine of rats during the initial 24 hr, of which only 15% were precipitated by TCA. Following heterologous ^{125}I- Fab administration in rabbits, guinea-pigs and mice, Spiegelberg & Weigle (1965) were able to recover 73–97% of administered ^{125}I, whereas only 4–46% could be recovered in TCA-precipitable form. The latter results were interpreted to indicate *in vivo* catabolism. Janeway *et al.* (1968) also concluded that *in vivo* catabolism in man was extensive since most of the radioactivity excreted by their two subjects was not protein bound.

A primary role of the kidney in catabolism of small molecular weight proteins has been suggested by studies of immunoglobulin light-chain metabolism in uremic subjects (Solomon *et al.*, 1964) and in nephrectomized mice (Wochner *et al.*, 1967). Also, following nephrectomy in rats, Fab elimination half-life was prolonged eight- to nine-fold whereas following maleate-induced renal tubular damage renal Fab excretion was enhanced without change in elimination half-life (Arend & Silverblatt, 1975). Further, Mogielnicki, Waldmann & Strober (1971) have shown that renal tubular damage increases the rate of renal elimination of immunoglobulin light chains in mice. Earlier studies indicated that filtered low molecular weight proteins are taken up by cells of the proximal tubule (Oliver, MacDowell & Lee, 1954;

Lathem *et al.*, 1960). This important role of renal catabolism clearly warrants consideration when Fab is administered in subjects with impaired renal function. The larger molecular weight IgG, on the other hand, was not present in appreciable amounts in the urine in the current study, in agreement with earlier observations (Waldmann & Strober, 1969; Andersen, 1964).

The present findings provide a basis for several earlier observations. Butler *et al.* (1977) administered digoxin-specific Fab or IgG to dogs 2 hr after a 0·02 mg/kg dose of digoxin. Plasma digoxin levels rapidly increased indicating a redistribution of digoxin from the extravascular to the intravascular compartment. The maximal plasma elevation observed in Fab-treated dogs, however, was considerably less than that seen in IgG-treated dogs, and fell much more rapidly. We have made similar observations in dogs and Rhesus monkeys given specific IgG or Fab after digitoxin administration (Ochs & Smith, 1977). The differences in IgG and Fab distribution and elimination observed in the present studies correlate well with these earlier findings.

On theoretical grounds, the smaller and more rapidly excreted heterologous Fab fragment population, lacking some of the species-specific antigenic determinants of the Fc fragment, might be expected to be less antigenic than the parent IgG molecule. There are, however, few data available that bear specifically on this point. Logistic and financial constraints limit the ease with which such studies can be carried out in primate species. The immunogenicity data obtained in baboons, although suggesting greater immunogenicity of intravenously injected sheep IgG than of Fab, do not permit any firm conclusions to be drawn. Techniques were developed, therefore, to permit the quantitative assessment of immune responses to sheep IgG and Fab in the rabbit. The method used was conceptually similar to that of Cerottini (1968), except that an additional antibody was used rather than ammonium sulfate at 40% saturation to ensure precipitation of soluble antigen–antibody complexes. The need for methods that detect soluble complexes in studies of this sort is evident from the work of Cerottini (1968) and Speigelberg & Weigle (1967).

The results of the immunogenicity studies in the rabbit clearly demonstrate a delayed and smaller immune response to intravenously administered heterologous Fab fragments, compared with the parent IgG population, in this species. It should be borne in mind that significant amounts of antibody of the IgE class could be formed but would escape detection with the methods used. It is reassuring in this regard that none of the baboons or rabbits tested showed any signs of anaphylactic reaction, even after repeated challenges with antigen.

In conclusion, in contrast to IgG, Fab fragments are distributed in the body rapidly and extensively after intravenous administration, and undergo substantial early catabolism and excretion. Taken together with their lesser antigenicity, these properties suggest potentially important advantages over IgG for use as therapeutic agents in selected clinical circumstances in man. The observed Fab elimination half-life of 9–13 hr in a primate model is appreciably longer than earlier estimates in small animals and, together with the other data presented here, should be taken into consideration when formulating Fab fragment dosing regimens in man.

We are indebted to Ms Elaine Havens and to the staff of the New England Regional Primate Research Center for technical assistance.

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ANTIBODY PURIFICATION PROCESS AND PRODUCTS

ABSTRACT

F(ab) fragments are isolated from an antibody containing source by contacting the antibody containing source with a papain-polyacrylamide matrix to produce F(ab) and F(c) fragments which are then passed through an antigen-polyacrylamide gel capable of attracting the F(ab) fragments. $F(ab)_2$ fragments are obtained by contacting the antibody containing source with a pepsin-polyacrylamide matrix to produce $F(ab)_2$ and F(c) fragments which are then passed through an antigen-polyacrylamide gel capable of attracting the $F(ab)_2$ fragments. IgG antibodies are obtained by passing an antibody containing source through an antigen-polyacrylamide gel. These processes can be used to purify a wide variety of antibodies which can be used as therapeutic agents and as diagnostic agents. Antivenins produced by these processes have substantially reduced foreign protein levels and hence are less likely to produce immunogenic reactions. Bulk, unprocessed antibody sources may be utilized and, for reasons of process simplification, are preferred.

FIELD OF THE INVENTION

This invention generally relates to processes for purifying antibodies. More specifically it relates to antibody purification by affinity chromatography processes using polyacrylamide gels.

BACKGROUND OF THE INVENTION

It is well known that antigen-polyacrylamide gels can be used to entrap antibodies. It is also well known that certain enzymes, e.g., trypsin, amylase and ribonuclease have been entrapped and used as attractive antigens in such gels (Science 142:678-679. 1963). Antigens can also be purified in a similar manner using specific antibodies

in the acrylamide gels to attract the antigens (Journal of Histochemistry and Cytochemistry, 23(2):146-148, 1975). These tendencies for certain antibodies and antigens to attract one another can be put to practical use. For example, an affinity chromatography procedure using a venom-polyacrylamide gel has been used to further purify equine source antivenin (Proc. West. Pharmacol. Soc. 25:185-192, 1982). That is to say, venom-polyacrylamide gels have been used to further purify a commercially available antivenin, Antivenin (Crotalidae) Polyvalent ("ACP"-Wyeth Laboratories, Box 8299, Philadelphia, Pennsylvania, U.S.A.) which is normally purified by ammonium sulfate precipitation procedures (American Journal of Emergency Medicine, 1:83-93, 1983). It is also well known that various enzymes such as trypsin, chymotrypsin and papain can be used to split antibody molecules into fragments having higher activities and lower toxicities than the original antibody molecule (see for example, U.S. Patent 4,012,502). Such procedures normally involve removing desired antibody containing protein fragments in a fractionating column, solution digesting these antibody containing protein fragments with various enzymes and then isolating the sought after antibody fragments in Sepharose columns.

Conceptually, a whole antibody molecule, commonly referred to as IgG, is often thought of as being comprised of three fragments connected in a Y shape. The two upper fragments are each referred to as F(ab) fragments. The stem of the Y is commonly referred to as the F(c) fragment. Each of the F(ab) fragments can be split from each other and from the F(c) fragment. The whole antibody, IgG molecule can also be cleaved into a larger fragment commonly referred to as a $F(ab)_2$ fragment and a F(c) fragment. This cleavage takes place in such a manner that an $F(ab)_2$ fragment is comprised of two attached F(ab) fragments. It is well known in the art that exposure of IgG molecules to papain produces F(ab) fragments and that pepsin digestion produces $F(ab)_2$ fragments. It is also well known in the art that the smaller F(ab) fragments are less likely to cause undesired immunogenic reactions. A

general rule is that, given possession of the antibody active site, the smaller the antibody molecule the better.

IgG, F(ab) fragments and $F(ab)_2$ fragments often have separate utilities. Furthermore, F(ab) and $F(ab)_2$ fragments may sometimes be utilized together. Hence processes for obtaining antibodies in each of these forms are highly desirable. For example, F(ab) fragments have the same affinity for antigens as IgG molecules, but they have lower molecular weights. Consequently, for reasons hereinafter more fully discussed, they can be more quickly distributed in the body and then filtered and excreted by the kidney. IgG molecules on the other hand are generally too large to be excreted by kidney functions. This means that whole IgG cannot distribute to tissue sites and neutralize toxins similar to F(ab) nor can the IgG and toxin combination be excreted by kidneys. However, IgG molecules can perform other useful functions. The F(c) portion of their molecule normally acts as a signal marker for lymphocytes to recognize and phayocytize. This is the chief pharmacological reason for leaving an IgG molecule intact. If for example a sought after antigen molecule is already a large molecule, the use of F(ab) fragments is limited. Even though the F(ab) fragments can be used to neutralize large antigen molecules, the kidneys still will not be able to excrete them. In such cases it may be better to use a whole IgG molecule to find the large antigen molecule so that the entire molecule assembly is phagocytized. See for example, Clin, Exp. IMMUNOL, 36:384-396(1979).

Another advantage to having the ability to break IgG antibodies into F(ab) or $F(ab)_2$ fragments is found in the pharmacological concept of volume of distribution. Volume of distribution is that volume of the body in which a given drug is dissolved. Circulating blood has a certain volume, but the body's water volume is much greater. For most IgG proteins, the volume of distribution is limited to the volume of circulating blood. However, for smaller molecules such as F(ab) fragments, the volume of distribution may be the total volume of body water. Furthermore, smaller molecules such as F(ab) fragments

often have the ability to cross certain physiological systems and barriers such as, for example, the blood/brain barrier. Larger antibodies do not have this capability. Consequently, neurotoxins may not be accessible to IgG molecules since the IgG molecules are confined to the circulating blood system. However many neurotoxins, including some snake venoms, may be accessible to F(ab) fragments because F(ab) fragments usually have a volume of distribution which includes water; hence the F(ab) fragments may be capable of crossing the blood/brain barrier in both the incoming and outgoing directions. In such cases F(ab) fragments may be used to excrete many kinds of neurotoxins which are not otherwise accessible to IgG antibodies. Thus from both the pharmacological and toxicological point of view, the ability to separate and purify a given antibody into selected fragments is of great significance to its application.

Antibody purity is a particularly important issue in the antivenin preparation art. Antivenin is a suspension of venom-neutralizing antibodies prepared from the serum of animals (typically horses) hyperimmunized against a specific venom or venoms. Horse and other animal serums are often digested with pepsin to obtain antivenin agents which are then precipitated out of the solution. Monovalent Bothrops (Laboratories "MYN", S.A., Av. Coyoacan 1707, Mexico City 12, D.F., Mexico), Anticrotalic (Instituto Butantan, Ciaxa Postal 65, Sao Paulo, Brazil) and Centiviperin (Institute Pasteur d'Algerie, Rue Docteur Laveran, Alger, Algeria) are examples of antivenin antibodies which are pepsin digested and then precipitated with ammonium sulphate.

Unfortunately, such enzyme digestion and ammonium precipitation procedures do not remove all foreign proteins from horse serum derived antivenins. Consequently, some bite victims undergoing antivenin treatment suffer extreme life threatening allergic reactions to those foreign proteins which are not removed from the horse serum by prior art purification procedures. Less life threatening serum sickness reactions are also common. The exact mechanism for these allergic reactions

has not been elucidated. For example they do not appear to be precisely related to previous exposure to horse antigen. Many researchers believe they may be due to anticomplementary activity of the serum. Another school of thought takes the position that the purity of the antivenin rather than the origin of the serum is the more critical factor in such allergic reactions. As a precaution however, all patients with known allergies to horses or horse serum are normally regarded as being at risk for serious anaphylactic reactions if given horse serum antivenin without adequate preparation. Therefore, any new and more efficacious procedures for purifying antibodies in general should be considered as being particularly important to the antivenin preparation art.

SUMMARY OF THE INVENTION

This invention discloses the use of enzymoabsorbed affinity methods combined with reversible immunoabsorption chromatography procedures to produce and isolate F(ab) and $F(ab)_2$ fragments to any antigen. The overall procedure is, however, especially useful in producing and isolatig F(ab) and $F(ab)_2$ fragments to snake venoms as well as other venoms. Another disclosed immunoabsorption procedure can be utilized to produce a highly refined IgG antibody from a bulk antibody source. Furthermore, the processes of this invention are capable of producing antibodies without recourse to certain precipitation procedures employed in the prior art. The F(ab) fragments, $F(ab)_2$ fragments and IgG thus produced are more highly purified, have greater antibody activities and are less likely to produce immunogenic reactions than their counterpart antibodies produced by processes utilizing such precipitation procedures. Furthermore, these antibody fragments can be products from monovalent, polyvalent and monoclonal sources. For example, F(ab) fragments can be produced by digesting either polyvalent IgG(T) or polyvalent anti-horse serum by methods wherein the papain is not bound to the polyacrylamide a well as by methods wherein the papain is bound to polyacrylamide as

an enzymatic affinant. In a preferred embodiment of this invention each of the above antibodies is produced from bulk antibody sources.

The overall test procedures used to establish this invention began with an enzyme digestion of the antibody source. First, a papain-polyacrylamide matrix or a pepsin-polyacrylamide matrix was established to receive the antibody containing source. Again, the antibody containing source may vary considerably. For example, in the context of antivenin purification, the antibody source may be bulk, unprocessed hyperimmune equine serum, laboratory produced monoclonal antibodies or commercially available antivenins. Those skilled in the art will appreciate that F(ab) fragments (papain digest) or $F(ab)_2$ fragments (pepsin digestion) and their respective F(c) fragments can be produced from many other kinds of antibody sources. For example, the processes of this invention were also specifically employed to isolate hemagglutinin specific antibody to an influenza virus. The literature also suggests that purification of many other antibodies by use of the processes of this invention can be accomplished since specific antibodies to kidney basement membrane antigens, to human chorionic gonadotropin, to bovine serum albumin and to human immunoglobulin A each have been produced by analogus purification techniques. Antigens also can be isolated by these procedures since entrapped antibodies can be used to attract the antigens (see generally, J. Histochem. Cytochem. 23(2):146-148, 1975). However, this method is less preferred since, when this is done, much more antibody must be entrapped to ensure that available antigenic sites are on the surface of the gel fragments. Moreover, human IgG also can be entrapped and used to isolate anti-IgG antibodies (see generally, Nature, 221:385-386, 1969).

Other variations of this invention are also possible. For example, antibodies to venom proteins have been immobilized on other matrices, such as cyanogen bromide activated sepharose and used to isolate specific venom proteins (Period. Biol. 80, Supp. 1: 97-100 (1978).

However, heretofore, enzymes such as papain and pepsin have not been used to digest whole IgG to their relevant F(ab) and $F(ab)_2$ fragments which are then collected by affinity chromatography procedures. Furthermore, for reasons hereinafter discussed, the fact that such antibodies can be directly obtained from bulk or monoclonal sources is particularly noteworthy. Regardless of the source of the antibody, however, the purification process of this invention continues by capturing antibodies on an antigen-polyacrylamide affinity chromatography gel which has embedded within its matrix, an antigen with an affinity for the sought after antibody. In the alternative, an affinant having an affinity for the F(c) fragments may also be employed.

To prove the efficacy of our process, the captured antibodies were then separated from the polyacrylamide and compared to counterpart fragments or IgG produced from comparable antibody containing sources, which were produced by other purification procedures. For example F(ab) fragments produced directly from bulk, unprocessed hyperimmune equine serum by the process of this invention were compared by immunoelectrophoresis diagrams to F(ab) fragments produced from commercially available equine serum. As previously noted, these serums are purified by known ammonia sulfate precipitation procedures. The commercial serums were then subjected to papain digestion and affinity chromatography procedures to produce F(ab) fragments which are compared to the F(ab) fragments produced directly from the bulk source without the ammonium sulfate precipitation step normally employed in the production of the commercial antivenin. Thereafter, the F(ab) fragments produced by the process of this invention were also compared to the commercial antivenin with respect to their relative lethality-neutralizing abilities. These tests clearly established that the F(ab) fragment antibodies and IgG produced by the processes of this invention afford better protection against venom-induced pathophysiology than the commercial antivenin on a milligram per milligram basis.

Those skilled in the art will appreciate that when

pepsin, rather than papain, is embedded into the acrylamide matrix, $F(ab)_2$ rather than F(ab) fragments are obtained. Again bulk, monoclonal and partially purified commercial antibody sources may be used. Monovalent and polyvalent fragments can be produced. Such $F(ab)_2$ fragments should also afford greater protection against venom-induced pathophysiology than the commercial antivenin. Similarly, they should produce less acute hypersensitivity reactions than those produced by the commercial antivenin.

Furthermore, according to the antigen-polyacrylamide affinity chromatography aspects of this invention, IgG antibody molecules also can be directly purified from bulk sources or monoclonal sources. They can be produced by passing a bulk antibody source or a monoclonal antibody source through an affinity chromatography system having an antigen-polyacrylamide matrix. This matrix attracts the IgG antibodies which can then be isolated from the gel matrix by procedures such as those found in the examples disclosed in later portions of this patent disclosure. Such IgG molecules are also largely characterized by the fact that they too are more efficacious than commercial antivenin and they too tend to produce fewer and/or less severe allergic reactions than those IgG molecules which have not been processed in the antigen-acrylamide affinity column system of our invention. The inventive aspect of this patent disclosure with respect to IgG molecules produced in this way is the fact that IgG can be pulled directly from bulk sources and not just refined from partially purified sources as taught in the previously noted Proc. West. Pharmacol. Soc. article.

In a variation of the process taught by this invention, F(ab) fragments are produced by passing an antibody source (bulk, monoclonal or partially purified) through a first and a second affinity chromatography system. The first affinity chromatography system has a papain-polyacrylamide gel matrix. This system also splits IgG molecules within the antibody source into F(ab) fragments and F(c) fragments. The effluent from the first affinity chromatograph system is then fed into the second

affinity chromatography system which has an antigen-polyacrylamide gel matrix. This matrix attracts the F(ab) fragments which can then be isolated from the gel matrix by known procedures hereinafter described. Here again, the antigen embedded in the polyacrylamide gel matrix can be varied greatly to obtain other useful results. For example, when whole influenza viris was used as the embedded affinant; our process isolated a hemagglutinin specific antibody and enriched it from 1,000 to 2,000 fold over a common antisera preparative process. The HAI titer was enriched by a factor of two and the protein content decreased 500 fold.

As previously noted, an antibody rather than the antigen can be embedded in an acrylamide matrix to pick up a relevant antigen. This fact can be utilized in designing an immunodetection system which can also be used in the context of envenomation. For example plasma can be passed over such a column to determine if antibodies are being produced by the victim. The kind and degree of such antibody product can form the basis of such an immunodetection. Likewise the F(c) fragment, rather than the F(ab) fragment may be captured by an appropriate antigen affinant.

Furthermore, $F(ab)_2$ fragments may be produced by passing an antibody source (bulk, monoclonal or partially purified) through a first and a second affinity chromatography system. The first affinity chromatography system will have a pepsin-polyacrylamide gel matrix. This matrix splits the IgG molecules within the antibody source into $F(ab)_2$ fragments and F(c) fragments. The effluent from the first system is then fed into the second affinity chromatography system which has an antigen-polyacrylamide matrix. This matrix attracts the $F(ab)_2$ fragments which are then isolated from the gel matrix.

In a representative example of this process, F(ab) antivenin fragments are produced by contacting bulk, unprocessed hyperimmune equine serum (i.e., untreated by any solution enzyme digestion or ammonium sulphate precipitation processes) with a papain-polyacrylamide matrix prepared as follows. An acrylamide monomer (16%

acrylamide, 4% N, N-methylene-bisacrylamide)/papain mixture is polymerized by the addition of 0.4% ammonium persulfate in water and TEMED (N,N,N-N-tetramethylenediamine). The mixture is well mixed and a water layer used to exclude oxygen. After polymerization, the papain-polyacrylamide matrix thus formed is fractionated by forcing the gel through a small porosity stainless phosphate buffered saline (pH 7.4) until all fines are removed. The gel is then packed into a column and washed alternately with PBS and 0.1M glycine-HCL (pH 2.5). During this washing the effluents are monitored with a spectrophotometer (280 nM). The washing cycle is continued until a baseline reading is obtained on the spectrophotometer. The matrix-papain fractionated gel is then placed into a beaker with a papain solvent (0.5 M phosphate pH 8, 0.002 M EDTA, and 0.01 M cysteine) containing cysteine that activates the enzyme. Bulk, unprocessed hyperimmune serum is then stirred in with the papain-polyacrylamide fractionated gel. This stirring is at room temperature but can be done at 37° C to speed the digestion. Initial experiments have demonstrated that digestion of antisera begins soon after the serum is introduced and can be nearly completed in about four hours at room temperature. The papain digestion produces F(ab) fragments from the antivenin. The resulting mixture containing these F(ab) fragments is then introduced into an affinity chromatography column employing rattlesnake venom as the (affinant) antigen. The venom is embedded in the polyacrylamide by a polymerization process which is substantially the same as the process just described with respect to the formation of the papain-acrylamide gel. An initial peak eluted with PBS consists of foreign protein. After the effluent is returned to baseline, the solvent is changed to glycine-HCl. A second peak is eluted which consists of purified F(ab) antivenin fragments which are collected separately and thereafter used as therapeutic agents.

The specificity of the F(ab) fragments produced from the bulk, unprocessed hyperimmune equine serum is then compared, by immunoelectrophoresis methods hereinafter

described, to the F(ab) fragments produced from a commercial antivenin which was further purified by the affinity chromatography process taught in the previously cited Proc. West. Pharmacol. Soc. publication. The F(ab) fragments from these two different sources are the same. Hence applicants have negated the need for the ammonium sulfate precipitation procedures used in producing commercial antivenins. The F(ab) and $F(ab)_2$ fragments produced by this invention are believed to be proteins or polypeptides. The F(ab) antivenin fragments thus produced have a molecular weight of about 50,000 and the $F(ab)_2$ fragments have a molecular weight of about 100,000. The IgG molecules derived from bulk sources have a molecular weight of about 150,000. Lethality and inhibition experiments were then conducted to compare the antivenin activity of the F(ab) fragments to the antivenin activity of commercial antivenin. These experiments indicate that the F(ab) fragments have a far greater antivenin activity than antivenins produced by the ammonium sulfate precipitation procedures used in the production of commercial antivenins. Furthermore, this increased activity can be achieved with fewer and less severe allergic reactions since the active site remains on the F(ab) fragment while portions of the original protein molecules which are antigenic to humans are removed by the processes of this invention. A reduced level of immogenic reaction with respect to the IgG is demonstrated in applicants' previously cited Proc. West. Pharmacol. Soc. publication.

DESCRIPTION OF THE DRAWING

Figure 1 depicts immunoelectrophoresis diagrams of various forms of IgG.

Figure 2 depicts immunoelectrophoresis of 4 hour and 48 hour digests of F(ab) antibody materials.

Figure 3 depicts immunoelectrophoresis of whole equine serum.

Figure 4 depicts immunoelectrophoresis of 4 hour digests of F(ab) antibody materials.

Figure 5 is an elution diagram of the materials eluted from an antigen-polyacrylamide affinity column.

Figure 6 is a schematic presentation of the relationship of the processes of this invention.

Figure 7 depicts immunoelectrophoresis of 18 hour digest of F(ab) antibody materials from a polyvalent source.

Figure 8 is a schematic representation of the application of the process of this invention of a polyvalent or monovalent antisera.

DETAILED DESCRIPTION OF INVENTION

Papain-Polyacrylamide Matrix Preparation

A representative affinity papain-polyacrylamide matrix is prepared by adding 1500 ml of soluble papain (Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo., 63178), to 20 mls. of acrylamide and bisacrylamide monomer (27 mg/ml protein; 24 units/ug protein; 30.5 mg total protein; 732 units total in 20 mls.) Those skilled in the art will appreciate that other matrix forming materials could be used in the practice of this invention; however, acrylamide is highly preferred since it costs less than most other similar materials, it polymerizes quickly, its protein retention and efficiency are high, it can be fractionated easily to provide a uniform matrix and it is reusable and stores easily. In any event, after the papain is dissolved in the monomer solution, polymerizing agents, N, N, N-N-tetramethylethylene- diamine (TEMED) and 0.4% ammonium persulfate are added. After being formed, the resulting matrix is fractionated by forcing it through a stainless steel screen having a pore dimension of 0.3 mm. The fractionated matrix is washed 5 times with phosphate buffered saline (PBS) to rid the matrix of nonusable fine particulate matter. The matrix is then washed with alternating solutions of PBS and 0.1 M glycine (pH2.5) and monitored with a 280 nM ultraviolet detector. This assures that any extraneous papain or other foreign materials are removed. Preferably, the

papain/polyacrylamide matrix is immersed in a buffer solvent comprised of 0.5M phosphate (pH 8.0); 0.002M EDTA; and 0.01M cysteine. The immersed matrix is then ready to receive an antibody source. Twenty mls. of bulk hyperimmune equine serum is added to a beaker containing the papain-acrylamide buffer mixture and stirred for four hours (digestion periods from about 1 hour to about 48 hours are preferred and periods of from about 4 to 8 hours are most preferred). The resulting F(ab) containing solvent digest is then separated from the papain-acrylamide matrix by affinity chromatography. The digest is then passed over through representative antigen-polyacrylamide affinity chromatography columns prepared as follows.

Antigen-Polyacrylamide Column Preparation

Representative antigen-polyacrylamide gels used in establishing the antivenin aspects of this invention were prepared from venom taken from four rattlesnake species Crotalus atrox, C. adamanteus, C. scutulatus scutulatus and C. viridis helleri. Each sample was prepared in a separate column so that each venom could be tested as an affinant. One hundred mg of each venom were dissolved in separate 10 ml portions of acrylamide monomer (16% acrylamide, 4% N, N-methylenebisacrylamide) in PBS, pH 7.4, in a small beaker. Polymerization of the venom-acrylamide mixtures were achieved by addition of 500ul of 0.4% ammonium persulfate in water and 50ul of TEMED. The mixture is mixed well and water was layered over the surface to exclude oxygen. The venom-acrylamide mixture gelled in 10 minutes and was fractionated by forcing the broken pieces through a stainless steel mesh (0.3mm). After fractionation the venom-polyacrylamide gel was reduced to a particle consistency. This fractionated gel was defined 5-6 times with PBS and packed by gravity into columns 1cm X 20cm. The venom-polyacrylamide columns were washed with alternating cycles of PBS and 0.1 M glycine, pH 2.5 (0.1 M glycine, 0.154 M Na Cl, pH adjusted with HCl) until a steady baseline was obtained by

monitoring the effluent at 280nM with a spectorphotometer. The column was returned to pH 7.4 with PBS and was then ready to receive the solution containing F(ab) fragments produced by the papain-polyacrylamide digestion previously described. The solution containing the F(ab) fragments is passed through the column to attract the F(ab) fragments to the antigen (venom). The solution, diluted to 10 mls, was added with a PBS mobile phase. The effluent flow was monitored at 280 nM ultra violet detection. The results of this elution are depicted in Figure 5. An initial peak I eluted with PBS is extraneous protein. Peak II is eluted with 0.1 M Glycine, pH 2.5, after baseline stabilization. This peak II contains the F(ab) material. This material can be collected and the pH of the system restored to 7 with Tris buffer. The collected material is then dialyzed against distilled water (approximately 24 hours) and lyophilized for storage at -20°C.

Affinity Isolation of IgG

Columns prepared by the procedures taught in the above discussion, i.e., "Antigen-Polyacrylamide Column Preparation", can also be used to isolate IgG from bulk sources. PBS was used as the initial solvent. A first peak similar to that in Figure 5 contains extraneous protein. After the baseline was re-established, 0.1M glycine-HCl, pH 2.5 solvent was used to elute off a second peak which contains the IgG. The pH of the IgG antibody effluent is adjusted to 7.4 with TRIS buffer. The isolated, purified antibody effluent is dialyzed against distilled water for 24 hours at 10°C, lyophilized and stored at -20°C. This IgG antivenin can be isolated to each of the four venoms tested.

Again it should be noted that the previously cited Proc. West. Pharmacol. Soc. article teaches that such antigen-polyacrylamide affinity columns can be used to further purify commercial antivenin produced by ammonium sulfate precipitation procedures. However, in view of the problems of competitive reactions of proteins, and protein affinity for like proteins, it is surprising that this antigen-polyacrylamide column also has the ability to pull otherwise untreated IgG directly out of a bulk source such as a hyperimmune serum. A schematic relationship showing each of the steps of the processes of this invention, as applied to antivenin purification from a bulk source, is depicted in Figure 6.

Derivation of F(ab) Fragments from ACP

In order to determine that F(ab) fragments produced by the processes of this invention are similar to the F(ab) fragments which can be derived from commercial antivenin, the following test procedure was utilized. First, Wyeth Antivenin (Crotalidae) Polyvalent (ACP) was purified by the affinity chromatography processes taught in the previously noted Proc. West. Pharmacol. Soc. [illegible]. The resulting IgG antibodies are then digested by the papain-polyacrylamide digestion procedures taught

in the "Papain-Polyacrylamide Matrix Preparation" section of this patent application. The resulting F(ab) fragment containing solution is then introduced into a venom-polyacrylamide affinity column prepared according to the procedures taught in the "Antigen-Acrylamide Column Preparation" section of this patent application. Again, a first PBS eluted peak contains extraneous protein materials. After re-establishing a baseline, 0.1M glycine HCl pH 2.5, is again used to elute off a second peak which contains F(ab) fragments. These first and second peaks are substantially identical to those obtained (see Figure 5) when F(ab) fragments are derived from bulk, unprocessed hyperimmune serum by the conjunctive use of the procedures described in the "Papain-Polyacrylamide Matrix Preparation" and "Antigen-Polyacrylamide Column Preparation" sections of this patent disclosure.

The F(ab) fragments from ACP are also compared with the F(ab) fragments from bulk sources on the basis of their immunoelectrophoresis diagrams. For example the immunoelectrophoresis diagrams of Figure 1, when taken in conjunction, show that the affinity purified ("AP") IgG is mainly IgG(T), the 4 hour F(ab) digest does not precipitate against IgG heavy and light chains, and that the IgG (AP) does not precipitate against heavy and light chains. The anti-IgG(T) sample is placed in this test to see how much anti-T the standard IgG has compared to an affinity purified IgG (designated "IgG(AP)"). Applicants' IgG(AP) is very heavy in IgG(T). Other immunoelectrophoresis tests have shown that when applicants' IgG(AP) is immodiffused against IgG(T), the results indicate that the IgG(AP) is solely IgG(T) and not IgG. Neither the IgG(AP) nor the 4 hour digest react against normal IgG of the horse serum heavy or light chain. The fact that our IgG(AP) reacts against the T chain, but not against the H and L, indicates that it is predominantly IgG(T). Therefore we are isolating the high affinity antibody.

Taken in conjunction, the immunoelectrophoresis diagrams of Figure 2 show that the 48 hour digest product and the 4 hour digest product each precipitate bands

against anti-$F(ab)_2$ but not against anti-F(c). No reactions against IgG heavy and light chains are evident. The reactions against the anti-IgG(T) are strongly evident. An anti-$F(ab)_2$ fragment shows a strong 48 reaction. A weaker 4 hour digestion reaction is shown. Taken in total, this evidence indicates that both the F(ab) fragments from the 48 digestion and the F(ab) fragments from the 4 hour digestion derive from the IgG(T).

The immunoelectrophoresis diagrams of Figure 3 show that when Wyeth antivenin (WPCA) is reacted against anti-whole horse serum there are multiple precipitate bands. This indicates that the Wyeth antivenin contains many different proteins which are removed by the processes taught in this patent disclosure. The IgG (AP) against anti-whole horse serum shows one clear band. The 4 hour digest shows the same clear band. These diffusions do not show any reactions against the IgG heavy and light bands because the antibody material is derived from IgG(T). On the other hand, the IgG does show a reaction. This also indicates that Applicants' antibodies are much purer than the Wyeth antivenin.

Again taking the immunoelectrophoresis diagrams in conjunction, Figure 4 indicates the results of another set of tests on the 4 hour digest antibody products of this invention. Again, another clear precipitate band against $F(ab)_2$ is shown. A weak F(ab) reaction is noted along with a slight hint of a F(c) reaction. This would seem to indicate that a 100% digestion to F(ab) fragments was not accomplished in the 4 hour digestion period and that anti-F(c) reacts against the IgG(T) antibody. It is known from the literature on horse immunoglobulins that IgG and IgG(T) share common determinants on their F(c) portion. Consequently our preferred digestion period is in the 4 to 6 hour range.

Moreover, applicants have found that papain bound to polyacrylamide gel (e.g., using 108 mg in 5 ml of acrylamide-bisacrylamide monomer and Type III papain) will digest horse polyvalent antisera to F(ab) and F(c)

fragments (this took 18 hours at 37° C at the above concentration) just as it digests horse monovalent antisera. Likewise, the papain digests polyvalent IgG(T) to F(ab) and F(c) fragments. Furthermore, the papain need not be bound to polyacrylamide.

The proof of this particular digestion was established by immunoelectro-phoresing (IEP) anti-IgG(T) antibody against (1) polyvalent IgG(T) standard, traditional digest (see generally, Nisonoff, A. Methods Medical Res. 101:134-141, 1964), (2) polyvalent IgG(T) non-digested, (3) polyvalent anti-horse serum, traditional digest and (4) polyvalent anti-horse serum papain-polyacrylamide digest. Referring to the IEP Key shown in Figure 7 we note that the standard IgG(T) IEP anti-IgG(T) shows one precipitin arc (i.e., #2 on the IEP Key). Polyvalent IgG(T) digested by a traditional method was immunoelectrophoresed against anti-IgG(T) shows two precipitin arcs corresponding to F(ab) and F(c) (i.e., #1 on the IEP Key of Figure 7). Polyvalent anti-horse serum digested 18 hours with papain/polyacrylamide at 37° C IEP against anti-IgG(T) shows two precipitin arcs corresponding to F(ab) and F(c) with some remaining IgG(T) (i.e., #5 on the IEP Key). Polyvalent anti-horse serum digested for 48 hours with papain bound to polyacrylamide at 37° C IEP against anti-IgG(T) shows partial digestion to F(ab) and F(c) with remaining IgG(T) (i.e., #3 on the IEP Key). A schematic relationship showing each of the steps of the process of this invention, as applied to a polyvalent or monovalent antisera, is depicted in Figure 8.

LETHALITY DETERMINATIONS

After establishing the intravenous LD_{50} for each venom, the following lethality determinations were used to establish the efficacy of various antivenins prepared by the processes of this invention. Individual solutions of the venom-acrylamide purified antibodies, as well as those of the Wyeth ACP used in the comparisons, were allowed to stand for 30 minutes before use in these lethality

determinations. Swiss-Webster mice, weighing 20-26 g, were administered the individual solutions by tail vein in volumes less than 100 ul. Results were interpreted at the end of 24 hours unless otherwise indicated. The F(ab) fragments and IgG issued in these lethality determinations were prepared by the processes previously discussed in this patent disclosure.

The first determination is a venom plus 1 Lethal Dose 99 (LD_{99}) of Crotalus atrox venom. Using the LD_{99} dosage, 3 of 9 mice treated with Wyeth ACP antivenin were alive at the end of the 24 hour period. When an IgG produced from a bulk, unprocessed hyperimmune equine serum by the IgG purification process of this invention was employed as the antivenin, 6 of 9 mice survived the 24 hour period.

TABLE 1: Crotalus atrox

I.P.

Venom + LD_{99} sample

(LD50 = 2.32 mg/kg)

	(24 hrs.)	
	Alive	Dead
Venom	0	5
Venom + Wyeth	3	6
Venom + (IgG)	6	3

Table 2 shows the results of using 2 times the LD_{50} dose (2.32 mg/kg) and 4 times an equal weight of an F(ab) fragments antibody prepared from bulk unprocessed hyperimmune equine serum. As noted, these F(ab) fragments are the result of a 4 hour papain digestion.

TABLE 2: Crotalus viridis helleri

I.P.

2 x LD_{50} x 4 x sample

(LD_{50} = 2.32 mg/kg

	(24 hrs) Alive	Dead
Venom	0	4
Venom + Wyeth	1	3
4 hr F(ab)	3	1

Table 3 compares 48 hour digestion F(ab) fragments, IgG and 4 hour digestion F(ab) fragment antibodies to Wyeth ACP antivenin on the dosage basis indicated.

TABLE 3: Crotalus viridis helleri

I.P.

2 x LD_{50} + twice that of the sample

(LD_{50} = 2.32 mg/kg

	24 (hrs) Alive	Dead
Venom	0	4
Venom + Wyeth	1	3
Venom + 48 hr F(ab)	4	0
Venom + IgG	4	0
Venom + 4 hr F(ab)	4	0

Table 4 shows the time elapsed until death when twice the LD_{99} dose is administered in conjunction with twice the LD_{50} test antibody material. Applicants are of the opinion that the reason that the F(ab) fragment antibodies (4 hour and 48 hour) significantly delayed the time of death beyond the time afforded by the IgG is found in the previously discussed concept of volume of distribution.

Since there is a dynamic relationship between the antibody and venom, some of the venom in the attaching and releasing dynamic relationship will diffuse out of the bloodstream because the venom has a volume of distribution similar to that of the F(ab) fragments. The IgG on the other hand is limited to the bloodstream. Consequently, the F(ab) can follow the venom as it diffuses and neutralize it in places beyond the blood stream.

TABLE 4: Crotalus viridis helleri

I.V.

Time-Study

LD_{99} x 2 x LD_{50} test material

(LD_{50} = 1.61 mg/kg)

Venom	15 min
	18 min
	19 min
	17 min
Venom + Wyeth	22 min
	18 min
	71 min
	50 min
Venom + IgG	90 min
	36 min
	48 min
	50 min
Venom + 4 hr F(ab)	106 min
	72 min
	122 min
	6 hr
Venom + 48 hr F(ab)	120 min
	6 hr
	120 min
	8 hr 55 min

The results of Table 5 indicate that in this experiment, the 4 hour digest F(ab) fragments gave the best protection and the ACP antivenin afforded no protection. In this case the efficacy of the IgG was equal to that of the 48 hour digest F(ab) fragments.

TABLE 5: Crotalus viridis helleri

I.V.

2 x LD_{50} + 4 x sample

(LD_{50} = 1.61 mg/kg)

	#Mice	(24 hrs) #Alive	#Dead
Control	4	0	4
Antivenin (Wyeth)	5	0	5
Peak II, 48 hrs F(ab)	5	3	2
Peak II, 4 hrs F(ab)	5	5	0
IgG	5	3	2

Table 6 summarizes the time until death results of using a LD_{75} dosage against 2 times the LD_{50} antibody test material.

TABLE 6: Crotalus viridis helleri

I. P.

LD_{75} + 2 x LD_{50} test material

(LD_{50} = 2.32 mg/kg

Venom	84 min
	62 min
	4 hr
Venom + Wyeth	6 hr
	6 hr+
	8 hr
	8 hr
Venom + IgG	9 hr
	24 hr alive
	24 hr alive
	8 hr
Venom + 4 hr F(ab)	24 hr alive
	24 hr alive
	24 hr alive
Venom + 48 hr F(ab)	8 hr
	4 hr
	24 hr alive
	2 hr

The above data indicates that the F(ab) fragments as well as IgG prepared by the processes of this invention can be used in the treatment of human snake bite victims. The dosages will be adjusted to suit the particular circumstances of the envenomation. In any event, the antibodies produced by this invention have specific activities much greater than that of Wyeth antivenin. The purified F(ab) is believed to be a protein having a molecular weight of about 50,000 Daltons. Furthermore, the F(ab) fragments produced from bulk, unprocessed hyperimmune equine serum appear to be the same as the F(ab) fragments which can be produced from Wyeth ACP purified by ammonium sulfate precipitation procedures. The purified IgG is believed to be a protein having a molecular weight of about 150,000. These antibodies clearly retain their activities after the purification steps of this invention. Furthermore, the therapeutically active portion of these materials remains while other portions of the original protein molecule which are antigenic to humans are removed by the processes of this invention. Hence anaphylaxis in individuals sensitive to horse serum and serum sickness reactions in general should be significantly reduced by use of these antivenins.

Thus having described our invention, we claim:

CLAIMS

1. A process for isolating F(ab) fragments from an antibody containing source comprising: contacting the antibody containing source with a papain-polyacrylamide matrix to obtain a solution containing F(ab) and F(c) fragments; and passing the solution containing the F(ab) and F(c) fragments through an affinity chromacography system having a gel comprised of an antigen (having an affinity for the F(ab) fragments) embedded in a polyacrylamide matrix, whereby the F(ao) fragments are isolated from the F(c) fragments for subsequent recovery.

2. The process of claim 1 wherein the antibody containing source is a bulk, unprocessed hyperimmune serum.

3. The process of claim 1 wherein the antibody containing source is a monoclonal antibody source.

4. The process of claim 1 wherein the antibody containing source is partially purified by precipitation procedures.

5. A process for isolating F(ab) fragments from an antibody containing source comprising: contacting the antibody containing source with a papain-polyacrylamide matrix to obtain a solution containing F(ab) and F(c) fragments; and passing the solution containing the F(ab) and F(c) fragments through an affinity chromatography system having a gel comprised of an antigen (having an affinity for the F(c) fragments) embedded in a polyacrylamide matrix, whereby the F(ab) fragments are isolated from the F(c) fragments for subsequent recovery.

6. The process of claim 5 wherein the antibody containing source is a bulk, unprocessed hyperimmune serum.

7. The process of claim 5 wherein the antibody

containing source is a monoclonal antibody source.

8. The process of claim 5 wherein the antibody containing source is partially purified by precipitation procedures.

9. A process for isolating $F(ab)_2$ fragments from an antibody containing source comprising: contacting the antibody containing source with a pepsin-polyacrylamide matrix to obtain a solution containing $F(ab)_2$ and F(c) fragments; and passing the solution containing the $F(ab)_2$ and F(c) fragments through an affinity chromatography system having a gel comprised of an antigen (having an affinity for the $F(ab)_2$ fragments) embedded in a polyacrylamide matrix, whereby the $F(ab)_2$ fragments are isolated from the F(c) fragments for subsequent recovery.

10. The process of claim 9 wherein the antibody containing source is a bulk, unprocessed hyperimmune serum.

11. The process of claim 9 wherein the antibody containing source is a monoclonal antibody source.

12. The process of claim 9 wherein the antibody containing source is partially purified by precipitation procedures.

13. A process for isolating $F(ab)_2$ fragments from a bulk antibody containing source comprising: contacting the antibody containing source with a pepsin-polyacrylamide matrix to obtain a solution containing $F(ab)_2$ and F(c) fragments; and passing the solution containing the $F(ab)_2$ and F(c) fragments through an affinity chromatography system having a gel comprised of an antigen (having an affinity for the F(c) fragments) embedded in a polyacrylamide matrix, whereby the $F(ab)_2$ fragments are isolated from the f(c) fragments for subsequent recovery.

14. The process of claim 13 wherein the antibody containing source is a bulk, unprocessed hyperimmune serum.

15. The process of claim 13 wherein the antibody containing source is a monoclonal antibody source.

16. The process of claim 13 wherein the antibody containing source is partially purified by precipitation procedures.

17. A process for isolating IgG antibodies from a bulk, antibody containing source comprising: passing the bulk, antibody containing source through an affinity chromatography system having a gel comprised of an antigen having an affinity for the IgG antibody embedded in a polyacrylamide matrix, whereby the IgG antibody is isolated from the bulk, antibody containing source for subsequent recovery.

18. The process of claim 17 wherein the bulk, antibody containing source is bulk, unprocessed hyperimmune equine serum.

19. The process of claim 17 wherein the bulk, antibody containing source is a monoclonal antibody source.

20. An F(ab) fragment extracted from an antibody containing source according to the process of claim 1.

21. An $F(ab)_2$ fragment extracted from an antibody containing source according to the process of claim 9.

22. An IgG molecule extracted from bulk antibody containing source according to the process of claim 17.

23. An F(ab) fragment extracted from a polyvalent IgG(T) source according to the process of Claim 1.

24. An F(ab) fragment extracted from a polyvalent anti-horse serum according to the process of Claim 1.

25. An $F(ab)_2$ fragment extracted from a polyvalent IgG(T) source according to the process of Claim 9.

26. An $F(ab)_2$ fragment extracted from a polyvalent anti-horse serum according to the process of Claim 9.

27. An antivenin composition comprising an administrable form of F(ab) fragments which are active against venoms of species of the Crotalus genus, and which produce an electrophoresis showing that anti-$F(ab)_2$ materials give a precipitation band against the F(ab) fragments but produce no precipitation band against anti-F(c) materials and wherein said F(ab) fragments have a molecular weight of about 50,000.

28. An antivenin composition comprising an administrable form of $F(ab)_2$ fragments which are active against venoms of species of the Crotalus genus, and wherein said $F(ab)_2$ fragments have a molecular weight of about 100,000.

29. An antivenin composition comprising an administrable form of polyvalent F(ab) fragments which produce an electrophoresis showing that anti-$F(ab)_2$ materials give a precipitation band against F(ab) fragments but produce no precipitation band against anti-F(c) materials and wherein said F(ab) fragments have a molecular weight of about 50,000.

30. An antivenin composition comprising an administrable form of IgG molecules derived from a bulk antibody containing source which are active against venoms of species of the Crotalus genus and wherein the said IgG molecules have a molecular weight of about 150,000.

FIG. 1

IMMUNOELECTROPHORESIS
(I)

FIG. 2

IMMUNOELECTROPHORESIS
(II)

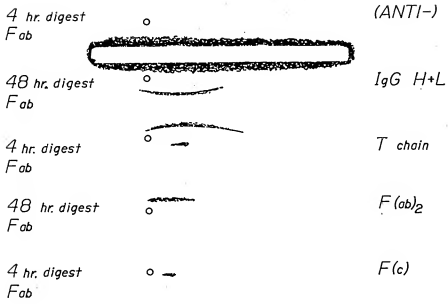


FIG. 3

IMMUNOELECTROPHORESIS
(III)

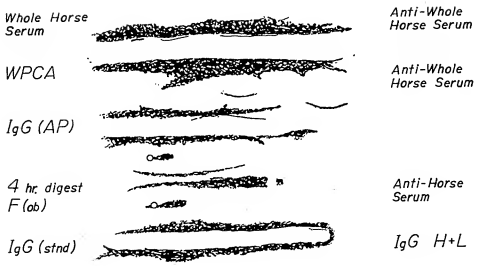
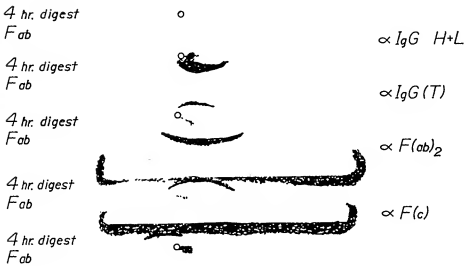


FIG. 4

IMMUNOELECTROPHORESIS
(IV)



CHROMATOGRAM OF F(ab) ISOLATION
BY AFFINITY COLUMN

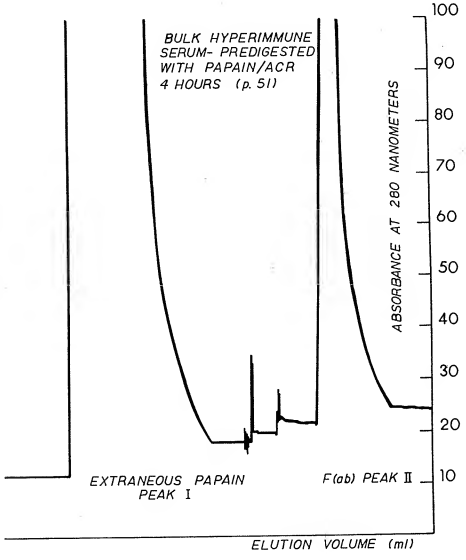


FIG. 5

FIG. 6

SCHEME OF PRODUCTION AND PURIFICATION SYSTEM

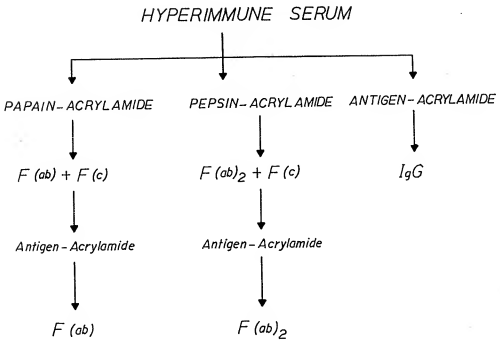


FIG. 7

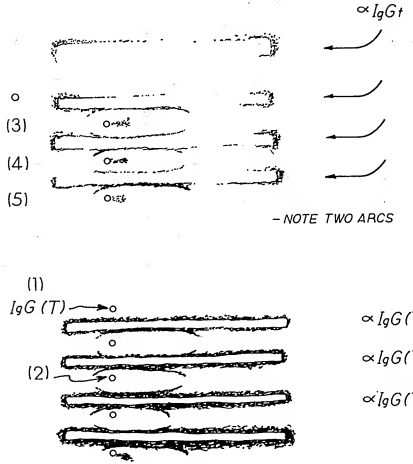
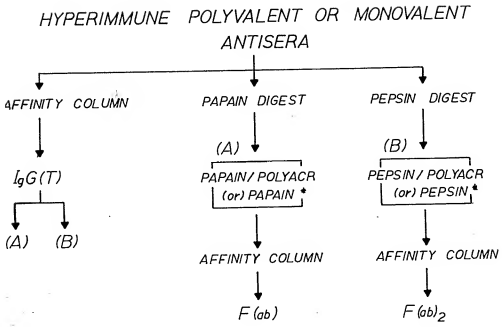


FIG. 8



* By modification of traditional method or by traditional method

SCHEME OF ISOLATION OF IgG(T), POLYVALENT AND MONOVALENT, AS WELL AS PRODUCTION AND ISOLATION OF ANTIBODY FRAGMENTS, POLYVALENT AND MONOVALENT. PROCESS CAN BE USED TO ISOLATE MONOCLONAL ANTIBODIES AND MONOCLONAL FRAGMENTS AS WELL AS ANTIBODIES AND FRAGMENTS OF ANTIBODIES TO ANTIGENS IMMOBILIZED IN THE POLYACRYLAMIDE. THE ANTIBODY CAN BE ISOLATED INITIALLY AND THEN DIGESTED BY EITHER A OR B, FOLLOWED BY FRAGMENT ISOLATION.

Am. J. Trop. Med. Hyg., 53(5), 1995, pp. 507–510

COMPARISON OF A NEW OVINE ANTIGEN BINDING FRAGMENT (Fab) ANTIVENIN FOR UNITED STATES CROTALIDAE WITH THE COMMERCIAL ANTIVENIN FOR PROTECTION AGAINST VENOM-INDUCED LETHALITY IN MICE

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Abstract. Snake venom poisoning is a medical emergency requiring immediate attention and the exercise of considerable judgment. Of the estimated 8,000 bites inflicted by venomous snakes in the United States each year, approximately 6,000 are treated with commercial antivenin. The only commercially available antivenin for North American Crotalidae envenomation is Antivenin (Crotalidae) Polyvalent (equine origin) (ACP; Wyeth Laboratories, Philadelphia, PA). A common complication is the high incidence of hypersensitivity reactions, occurring in more than 75% of patients treated with ACP. To minimize these side effects, a novel, affinity-purified, antigen binding fragment (Fab) antivenom (FabAV) for Crotalidae venom poisoning has been produced from the sera of sheep. The new product is Antivenin Polyvalent Crotalid (Ovine) Fab (Crotab®; Therapeutic Antibodies, Inc., Nashville, TN). The current report compares the potencies in mice of FabAV and ACP against venom-induced lethality. The results indicate that FabAV is 3.1–9.6 times more potent than ACP for the prevention of lethality of the nine United States venoms tested. For one of the venoms, *Crotalus viridis helleri*, FabAV was efficacious while ACP was not.

In the United States, there are approximately 8,000 bites by venomous snakes each year. Most of these bites are caused by Crotalidae, i.e., rattlesnakes, copperheads, and cottonmouths.[1–3] Between 1960 and 1990, 7–12 fatalities from snake venom poisoning were reported annually. Before the advent of antivenin (antivenom)* and antibiotics, it is estimated that the death rate from Crotalidae bites in the United States was approximately 5%, with mortality from secondary infections accounting for an additional 2%.[1–3] For example, records from the Klauber Library at the Zoological Society of San Diego give a fatality rate of 4–7% prior to the advent and availability of commercial antivenin in the United States. Klauber concluded that the most important factor for the reduction in the death rate was the early and adequate administration of antivenin.[4]

In spite of the effectiveness of antivenins in reducing mortality and morbidity, they have not been without serious shortcomings. The first antivenin for human use was prepared in horses against the venom of a cobra.[5] This and other early antisera were solutions of unrefined horse sera that frequently produced anaphylaxis, hypotensive crises, and numerous other deleterious reactions. The literature published between 1910 and 1930 indicates that these early antisera were sometimes as dangerous as the venom itself.[6] Through the intervening years, a number of improvements in antisera production have been made.[7] At present, most antivenins are either partially purified immunoglobulin G, (IgG), as produced in the United States, or antigen binding fragments, (Fab_2), as produced in Europe and other parts of the world. The former is associated with a higher degree of hypersensitivity than the latter.

The only commercially available antivenin for North American Crotalidae is Antivenin (Crotalidae) Polyvalent (equine origin) (ACP; Wyeth Laboratories, Philadelphia, PA). This antivenin may be associated with perhaps as high as a 15% immediate horse serum reaction and a 75% delayed horse serum response.[2, 8] Although many of the immediate horse serum reactions are minor, they often require changes in the antivenin administrative protocol, and on occasions have discouraged physicians from using antivenin therapy. The clinical importance of these finding is reflected in telephone calls to the Los Angeles County/University of Southern California Medical Center Venom Poisoning Center, and the Poison and Drug Information Center at the University of Arizona, in which approximately 20% of physicians' inquiries related to questions about reactions were associated with ACP (Russell FE, unpublished data). This amounts to more than 1,500 calls over the past 30 years.

Since horse IgG is sensitizing to humans, attempts have been made to produce a Crotalidae antivenin in rabbits and goats with seemingly with good results.[9, 10] In 1985, Russell and others published a report of several studies on antivenin purification using affinity chromatography.[11] The studies indicated that the technique removed extraneous proteins, including considerable albumin. Tests in mammals showed that the purified IgG did not elicit a sensitivity reaction, while the commercial ACP did.[11] A more quantitative study of affinity-purified ACP in guinea pigs demonstrated a non-sensitizing product.[12] Studies using polyacrylamide gel affinity-purified anti-Crotalidae antisera from the horse demonstrated that the purified material neutralized the lethal, cytolytic, hemorrhagic, platelet aggregating, and other deleterious effects of Crotalidae venoms, and that the purified antibodies gave no evidence of producing anaphylactic or anaphylactoid reactions in animals sensitized to horse se-

*The word antivenin was the identification used for the first antiserum for snake venom poisoning prepared for human use.[5] It has been retained in many parts of the world on the basis of historical precedent, but perhaps more importantly because it identifies a specific process, immunization, in its preparation. Antivenom, on the other hand, is frequently used for any product that is against a venom. e.g., trypsin and other chemicals.[6] The authors have chosen to use the word antivenin in this manuscript.

rum.[11] On the basis of these various experiments, the Department of Chemical Pathology at St. Bartholomew's Hospital Medical School in London instituted an effective immunization program in sheep followed by an extensive purification of the antisera. The resulting products were affinity-purified Fab directed against the venoms of four United States Crotalidae. The venoms for immunization were selected so that a blend of the final product would neutralize the lethal effects of all clinically important crotalid venoms of North American snakes. The material used in the present study, a novel, affinity-purified Fab antivenom (FabAV), was a blend of equal weights of the four Fabs. This study compares the efficacy of FabAV and ACP against crotalid snake venom–induced lethality in mice.

MATERIALS AND METHODS

Immunizing venoms. Four venoms were selected: *Crotalus atrox* (Western diamondback rattlesnake), *C. adamanteus* (Eastern diamond rattlesnake), *C. scutulatus scutulatus* (Mojave rattlesnake, venom A[13]), and *Agkistrodon piscivorus piscivorus* (Eastern cottonmouth). These venoms were chosen on the basis of 1) their clinical importance in snakebite cases in the United States and northern Mexico, 2) geographic range of the snake, 3) genetic dissimilarities of the four immunizing venoms, and 4) cross-antigenicity with venoms from other clinically important crotalids. The snakes were captured over their full range, and included newborn, juvenile, and adult specimens. All venoms were lyophilized within 24 hr of collection, pooled, homogenized, and stored in the dark at 5°C until the time of immunization or the venom-antivenin studies.

Immunization. The venom immunization program was carried out in sheep at Llandymul, Wales. The immunization was essentially the same as previously described.[14] Groups of sheep were injected with one of the four immunizing venoms. In the beginning, an enzyme-linked immunosorbent assay was used for the snake-specific IgG determinations following each monthly bleeding, but within several months it was observed that this test did not correlate with the in vivo efficacy of the antisera as determined in mice. Thereafter, the progress of the immunization program was monitored by the antisera protection of venom-induced lethality in mice. When the antisera of each of the four species proved to be more efficacious against their respective venoms than ACP, the sheep were bled for the present study. This occurred following the 18th month of immunization.

FabAV and ACP antivenins. The details of purification of sheep serum to snake-specific Fab have been described.[15] Briefly, the IgG of each of the four monospecific antiserum pools was precipitated with Na_2SO_4. The resulting IgG was redissolved, dialyzed, and digested with papain. The Fc was precipitated from the supernatant Fab at 4°C. Snake venom–specific Fabs were isolated by chromatography with the venom covalently coupled to cyanogen bromide–activated Sepharose 4B. Each monospecific Fab pool was affinity-purified against its respective venom, desorbed, dialyzed, and lyophilized. Equal weights of the species-specific Fabs were pooled to give FabAV.

The ACP was obtained from Wyeth Laboratories. While the ACP is packaged with a standardized neutralization equivalent in each vial, but the potency/mg of this antivenin varied with batch and lot. In the vials measured prior to the present study, the authors found that the amount of material varied from 0.8 to 2.4 g/vial. Since the former had the greater activity, it was chosen to compare with FabAV.

Test venoms. In addition to the four immunizing venoms, six other crotalid venoms were chosen for the antivenin neutralization experiments. As previously determined in our laboratory,[15] the venoms and their intravenous median lethal doses (LD_{50}s in mg/kg) in mice are *C. atrox* = 3.79, *C. adamanteus* = 1.35, *C. s. scutulatus* (venom A) = 0.17, *C. horridus atricaudatus* (Canebrake rattlesnake) = 0.92, *C. h. horridus* (Timber rattlesnake) = 6.32, *C. m. molossus* (Northern blacktailed rattlesnake) = 4.42, *C. viridis helleri* (Southern Pacific rattlesnake) = 3.48, *A. p. piscivorus* = 3.38, *A. c. contortrix* (Southern copperhead) = 4.99, and *Sistrurus miliarius barbouri* (Southeastern pygmy rattlesnake) = 4.87.

Venom-antivenin neutralization determinations. The neutralization experiments were carried out in female, 21–23 g, ICR stock, Harlan-Sprague Dawley mice. Six mice were housed in each cage and observed together throughout the quarantine period and experiments. Groups of six mice were challenged with a mixture of 2 × LD_{50} of venom and one of six graded doses of antivenin. The doses of antivenin included one that afforded no protection, one that protected the entire group, and four doses between these limits. In preliminary experiments, 2 × LD_{50} challenges of each of the 10 venoms were invariably lethal. Stock venom solutions containing 2 LD_{50} per 0.10 ml were freshly prepared at 0°C in 0.15 M NaCl and maintained no longer than 4 hr at 0°C before use. For each mouse, 0.10 ml of stock venom solution was mixed with 0.10 ml of antivenin. The mixtures were stoppered, incubated at 37°C for 30 min in a water bath, and then injected into the tail vein of a mouse. The injection volume was 0.20 ± 0.02 ml. Following injections, the mice were observed for 48 hr and the time to death or survival was recorded. The median effective doses (ED_{50}s), 95% confidence limits (CLs), and statistical comparisons of dose-response slopes and potency ratios, were calculated by the method of Litchfield and Wilcoxon,[16] as incorporated into a computer program by Tallarida and Murray.[17]

RESULTS

The ED_{50}s and 95% CLs of the two antivenins are shown in Table 1. In the experiments with ACP and *C. v. helleri* venom, only 67% protection from lethality could be obtained at the maximum solubility of the ACP. Thus, the ED_{50} could not be determined because 100% protection was unobtainable. Potency ratios (ED_{50} of ACP and FabAV) and their 95% CLs are also shown in Table 1. They range from 3.1 to 9.6, with a mean value of 5.1. These values reflect the ratio of the amount of ACP required to provide the same degree of protection from venom lethality as FabAV. For each of these comparisons, FabAV was significantly more potent than ACP.

DISCUSSION

The results of the present study indicate that FabAV is an effective antivenin in preventing lethality induced in mice

TABLE 1

Median effective doses (ED_{50}), potency ratios, and 95% confidence limits (CLs) of FabAV and ACP antivenins against two LD_{50} challenges of 10 United States Crotalidae venoms*

Venom species	FabAV†	ACP antivenin†	
	ED_{50} (95% CL)	ED_{50} (95% CL)	Potency ratio‡ (95% CL)
C. viridis helleri	849.8 (762.9–946.5)	–§	–
C. molossus molossus	217.7 (134.9–351.3)	680.4 (466.2–992.9)	3.1 (1.7–5.8)
C. horridus horridus	81.2 (61.6–107.2)	251.2 (135.7–465.0)	3.1 (1.6–6.1)
C. adamanteus	22.7 (13.4–38.3)	79.0 (62.2–100.4)	3.5 (2.0–6.2)
C. horridus atricaudatus	12.7 (8.0–20.3)	45.4 (32.3–63.8)	3.6 (2.0–6.4)
C. atrox	39.2 (20.6–74.3)	180.3 (120.0–271.0)	4.6 (2.2–9.8)
C. scutulatus scutulatus A	4.9 (4.1–6.0)	23.8 (17.8–31.9)	4.8 (3.4–6.9)
S. miliarius barbouri	70.1 (48.6–101.0)	413.1 (272.8–625.4)	5.9 (3.4–10.2)
A. contortrix contortrix	35.9 (29.8–43.3)	281.6 (198.5–399.5)	7.8 (5.3–11.6)
A. piscivorus piscivorus	21.9 (16.3–29.5)	211.4 (158.8–281.4)	9.6 (6.4–14.6)

* FabAV = ovine Fab antivenin (Crotalidae), ACP = antivenin (Crotalidae) polyvalent; LD_{50} = median lethal dose; *C. = Crotalus; S. = Sistrurus; A. = Agkistrodon.*
† Expressed as mg of antivenin/kg of mouse body weight; ED_{50} values were determined against 2 × LD_{50} of venoms (see Materials and Methods for details).
‡ For ED_{50} ACP/ED_{50} FabAV; each potency ratio was significant at $P < 0.05$.
§ The ED_{50} could not be determined because of the lack of maximal dose response protection.

for a wide variety of clinically important North American snake venoms. It is approximately five times more potent than ACP. Also, FabAV is more effective in preventing lethality in mice due to *C. viridis helleri* venom. Translated into clinical terms on the basis of the mouse potency data, this would indicate that only one-fifth as much antivenin might need to be given for the same degree of protection.

Although the present study did not formally assess the toxicity of the antivenins, no untoward reactions occurred in any of the mice tested with either antivenin. There are, however, several fundamental differences between FabAV and ACP that would predict a greatly reduced serum reaction for FabAV. The FabAV is derived from sheep serum in the production of the antivenin, while ACP is derived from horse serum. The FabAV is derived from four groups of sheep immunized with one of four venoms, while ACP is derived from one group of horses immunized with a mixture of four venoms. Four venoms of North American crotalids are used in the immunization program to produce FabAV, while two United States venoms and two Central and South American venoms are used in the production of ACP. The FabAV is derived from IgG precipitated with Na_2SO_4, while ACP is precipitated with $(NH_4)_2SO_4$. The FabAV is derived from IgG digested with papain, while ACP is undigested IgG.

The use of purified Fab should circumvent the allergenic and immunogenic properties of Fc and IgG, while the affinity purification of Fab reduces the total protein by eliminating nonspecific Fab and other sheep serum components not eliminated by previous purification steps. A similarly produced Fab against *Vipera berus* venom (prepared by Therapeutic Antibodies, Inc., Nashville, TN) has been used successfully in Europe without apparent side effects.[18] Additionally, clinical experiences with the antidigitalis drug, digoxin immune Fab (ovine) (Digibind; Burroughs Wellcome Co., Research Triangle Park, NC), indicates the considerable safety of sheep Fab products.[19,20] As a result of these findings and those described herein, a clinical trial of FabAV is in progress.

Acknowledgments: We thank Dr. M. J. Schumacher for helpful comments on the manuscript, and Dr. I. Rees and N. Bucknall for technical assistance.

Financial support: this was supported by a grant from Therapeutic Antibodies, Inc. (Nashville, TN).

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Efficacy, Safety, and Use of Snake Antivenoms in the United States

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Received for publication August 25, 2000. Revision received November 29, 2000. Accepted for publication December 5, 2000.

Presented at the North American Congress of Clinical Toxicology's "Advances in the Management of Snakebite" Symposium, October 1999, La Jolla, CA.

Dr. Dart and Mr. McNally were clinical investigators in the trials of the experimental antivenom described in the text and were paid speakers in the symposium "Advances in the Management of Snakebite" that was sponsored by Savage Laboratories.

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0196-0644/2001/$35.00 + 0
47:1/113372
doi:10.1067/mem.2001.113372

See related articles, p. 168, p. 175, p. 189, and p. 196.

The mainstay of hospital treatment for venomous snakebite is antivenom. There is currently only one antivenom available in the United States for the treatment of pit viper envenomation, Antivenin (Crotalidae) Polyvalent (ACP). The general indication for the administration of antivenom is presence of progressive venom injury. Progressive injury is defined as worsening local injury (eg, swelling, ecchymosis), a clinically important coagulation abnormality, or systemic effects (eg, hypotension, altered mental status). Unfortunately, there are no prospective data available regarding the efficacy of ACP. The efficacy of a new antivenom (CroFab; FabAV) composed of purified Fab specific to indigenous snake species has been demonstrated in prospective trials. FabAV appears as effective as IgG antivenoms. However, Fab molecules have a shorter half-life than IgG molecules and may allow recurrence of venom effects, if additional doses are not administered. It has also been found that other antivenoms, including ACP, also allow recurrence of venom effects. The Fab preparation has produced fewer acute or delayed (serum sickness) allergic reactions; however, further experience is needed to confirm this observation. Evaluation of this new antivenom has led to advances in our understanding of antivenoms in terms of solubility and durability. Fab fragments enter solution quickly, thereby shortening the time to antivenom administration and are remarkably stable under extreme conditions of heat and handling.

[Dart RC, McNally J. Efficacy, safety, and use of snake antivenoms in the United States. *Ann Emerg Med*. February 2001;37:181-188.]

INTRODUCTION

Snakebite is an infrequent but serious health problem in the United States. Approximately 6 deaths and several thousand venomous snakebites occur each year.[1,2] The primary snakes of concern in the United States are the pit

vipers: rattlesnakes, cottonmouth, and copperhead snakes. These snakes were previously categorized as the crotalid snakes (family Crotalidae). However, the classification for the pit vipers changed about 15 years ago, an event largely overlooked in clinical medicine. The classification nomenclature is now family Viperidae, subfamily Crotalinae; therefore, the more appropriate term "crotaline" is used to refer to the Crotalinae subfamily, which includes North American species of *Agkistrodon* (copperhead and cottonmouth snakes), *Crotalus* (rattlesnakes), and *Sistrurus* (massasauga and pigmy rattlesnakes).[3]

The history of snakebite treatment is long and colorful. Egyptian priests incised snakebites to "...let the evil spirits out."[4] In the early 1900s, whiskey was the preferred antidote but produced many side effects, some desirable. Sadly, the medical literature reveals that some deaths, particularly in children, were more likely the result of the alcohol forced on them than the serpent's bite.[5] Over the years, snakebite "experts" have recommended carbolic acid, strychnine, enemas, urine, cauterization, electric shocks, and many other imaginative techniques for the treatment of snakebite.

New medical developments converged in the 1960s to form the current treatment for snakebite. An antivenom, Antivenin (Crotalidae) Polyvalent (ACP), was introduced by Wyeth Laboratories in 1954 and in the following decade, emergency departments and critical care units were introduced. Each of these developments undoubtedly contributed to the remarkable decrease in mortality rate from crotaline snakebite that has occurred over the past century. It is estimated that mortality rates were 5% to 25% in the 19th century.[6] More recent estimates suggest that the mortality rate is less than 1% and probably less than 0.1% for patients treated with antivenom in a health care facility.[2,4,6]

Although it is the only antivenom available for the treatment of crotaline snakebite in the United States, no prospective randomized investigation of ACP has been performed. This fact, combined with the fact that general improvements in supportive care have accompanied the reduction in mortality, have created doubt that antivenom is effective.[7] This article defines the clinical challenges involved in the treatment of crotaline snakebite and summarizes the evidence available to guide the clinician in the use of antivenoms.

THE CLINICAL PROBLEM

Antivenoms have been developed for nearly all venomous snake venoms. Outside the United States, the efficacy and relative safety of antivenoms have been demonstrated in randomized trials.[8,9] In general, these trials have demonstrated that antivenoms are effective when a sufficient dose is administered at the appropriate time. In the United States, however, use of antivenoms has been questioned because of a hypothetical unfavorable risk/benefit ratio.[7,10] The primary risks associated with the use of antivenom are anaphylaxis and serum sickness. However, neither the risks nor benefits have been studied prospectively in the United States.

To assess the risks and benefits of antivenoms, it is crucial to fully understand 2 basic characteristics of venomous snakebite. First, snake venoms may cause both reversible and irreversible injuries. For example, coagulation abnormalities after crotaline snakebite are usually reversible.[11] The venom of most crotaline snakes contains components that can cause a coagulopathy by activating or inhibiting the activity of various coagulation factors in the victim.[12] If binding with an antibody neutralizes these venom components, the victim can quickly replace the coagulation components because permanent injury has not been caused and reserves exist in the spleen and bone marrow. In contrast, local tissue injury caused by crotaline venom typically fails to reverse quickly.[11] Crotaline snake venoms often cause marked cell swelling and increased vascular permeability, leading to pain, edema, and ecchymosis soon after envenomation. Localized cell death and tissue necrosis are also common.[13] In this instance, antivenom can halt further injury and may foster healing by inactivating venom factors, but it would not be expected to promptly reverse advanced cellular or tissue injury.

Another challenge is the dynamic nature of crotaline snake envenomation. Immediately after a bite, a crotaline snake envenomation is categorized as minimal by definition (fang marks and perhaps localized pain and swelling).[4] In many cases, the initially minimal injury may worsen to become limb- or life-threatening. The envenomation destined to become severe must therefore pass through the grades of minimal and moderate. Conversely, the venom effects may worsen in an indolent manner over many hours or the bite may be "dry," failing to worsen because no venom was injected.[4] The variable nature of venomous snakebite renders clinical evaluation difficult. If a therapy is applied to a bite that will not worsen (a dry bite), it may erroneously appear to be an effective treatment. In contrast, a massive envenomation could overwhelm all forms of therapy, causing an otherwise effective therapy to appear ineffective. Therefore, the problems of bias as well as incomplete and inaccurate data that are inherent to case

reports and retrospective chart reviews are particularly severe in the study of crotaline snakebite. Controlled comparative trials with appropriate selection of subjects is crucial.

EFFICACY OF ANTIVENOMS

There are now 2 antivenoms available for the treatment of crotaline snakebite (Table 1). Antivenin (Crotalidae) Polyvalent (Wyeth-Ayerst Laboratories) is a horse serum–derived antivenom. Crotalidae Polyvalent Immune Fab (Ovine) (Altana, Inc.) is produced from sheep serum and was approved by the US Food and Drug Administration in October 2000, after more than 15 years in development.

ACP has been an important part of snakebite therapy for 35 years. In animals, ACP uniformly prevents death after injection of a wide range of crotaline snake venoms; however, its efficacy diminishes if administration is delayed.[14,15] The presumption of efficacy for ACP is based on these animal studies, several retrospective studies, and numerous case reports. Nevertheless, observations and articles written by practitioners from a range of medical specialties support the concept that ACP is effective, particularly for the life-threatening or coagulopathic manifestations of crotaline snakebite.[16-21]

On the other hand, retrospective reports have also documented apparent therapeutic failures of ACP. These involve the inability of ACP to effectively treat thrombocytopenia in some patients, particularly in the case of the timber rattlesnake (*Crotalus horridus horridus*).[22] Failure to reverse the neurologic effects of Mojave rattlesnake venom has also been described.[23] Finally, severe tissue loss has been described despite the use of antivenom.[24,25] However, it is apparent in these reports that tissue loss was associated with the use of small doses of antivenom or delay in its administration, thereby allowing tissue necrosis to occur.

The new antivenom for crotaline envenomation, Crotalidae Polyvalent Immune Fab (Ovine) (CroFab, FabAV) is produced by immunizing flocks of sheep with 1 of 4 crotaline snake venoms (Table 1). The ovine immune serum from each flock is then digested with papain to produce antibody fragments (Fab and Fc), and the more immunogenic Fc portion of the antibody is then eliminated during purification. The 4 individual monospecific Fab preparations are combined to form the final antivenom product. When tested in an animal lethality model, the new antivenom averaged 5.2 times (range 3.0 to 11.7) more potent than ACP against 14 different crotaline snake venoms.[26]

In contrast to ACP, prospective data are available for FabAV. Two trials have been performed: a prospective observational pilot trial and a randomized comparative trial of 2 different dosage regimens.[27,28] Both trials enrolled patients 10 years or older within 6 hours of crotaline snakebite. Bites by the copperhead snake were excluded. To decrease the likelihood that the venom effects could subside spontaneously during antivenom treatment, both trials required that the patient be treated within 6 hours of envenomation and that evidence of progression be present at the time of enrollment. Both trials used a systematic assessment tool, the snakebite severity score (SSS), to document the severity of envenomation more objectively than previous studies.[11]

The open-label pilot trial involved 11 patients who were treated with an initial infusion of 4 to 8 vials of FabAV without further doses, much like the administration of ACP. The SSS of all patients improved after treatment. No acute or delayed allergic reactions to FabAV occurred. However, re-emergence of limb swelling was found in 3 (27%) of the patients. In addition, recurrence of hypoprothrombinemia was discovered in 1 patient at a 7-day follow-up visit. To prevent these recurrences, a new

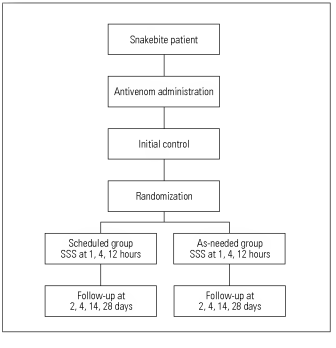
Table 1.
Comparison of Antivenin (Crotalidae) Polyvalent and Crotalidae Polyvalent Immune Fab (Ovine).

Variable	Antivenin (Crotalidae) Polyvalent	Crotalidae Polyvalent Immune Fab (Ovine)
Animal source	Horse	Sheep
Venoms used to immunize animal	*Crotalus adamanteus* (Eastern diamondback) *C atrox* (Western diamondback) *C durissus terrificus* (tropical rattlesnake) *Bothrops atrox* (fer-de-lance)	*C adamanteus* (Eastern diamondback) *C atrox* (Western diamondback) *C scutulatus* (Mojave) *Agkistrodon piscivorus* (cottonmouth)
Immunoglobulin (molecular weight)	IgG (150,000 daltons)	Fab (50,000 daltons)
Purification method	Ammonium sulfate precipitation	Sodium sulfate precipitation and affinity purification
Constituents		
Total protein	2.1 g/vial	<1.5 g/vial
Albumin	Albumin 120 mg/vial (6% w/w)	Albumin (<0.5% w/w)
Total antibody	IgG (18.9% w/w)	Fab (>85% w/w) Fc (<3% w/w)
Color	Yellow	White

dosing schedule was devised and an open-label randomized comparative trial of 31 patients that used the same entrance criteria as the pilot study was performed. The dosing of FabAV was different (Figure 1): each patient received an intravenous dose of 6 vials, which could be repeated once for a total of 12 vials to achieve initial control. Once initial control was achieved, those in the as-needed group received no further antivenom unless the patient worsened, whereas those in the scheduled group received additional 2-vial treatments with FabAV at 6, 12, and 18 hours after initial control was achieved.

Initial control of all manifestations of envenomation was achieved in all 42 patients with doses of FabAV ranging from 3 to 12 vials. One patient received only 3 vials of the initial 6-vial dose because of an acute antivenom reaction. The mean SSS began to decrease during the initial infusion of antivenom and continued through the 12-hour period of efficacy evaluation in both studies (Table 2). The decrease in the severity of illness as represented by the SSS was composed of decreases in the values for the coagulation, central nervous system, gastrointestinal, and cardiovascular components of the score (Figure 2).

Figure 1.
Study schematic for randomized trial of Crotalidae Polyvalent Immune Fab (Ovine).[28]



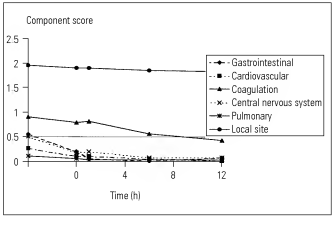
The values for each of these parameters decreased during the initial infusion of antivenom and continued to decrease throughout the evaluation period. Thus, venom-induced abnormalities in these organ systems appear to be reversible. In contrast, the local injury component of the score decreased only slightly and was not statistically significant. This observation may be explained by the fact that ecchymosis and edema formation involve hemorrhage, cell swelling, and cell death, processes that are irreversible or only slowly reversible.[1,3]

Table 2.
Summary of mean total severity scores for FabAV studies.

		Comparative Trial			
Study Period*	Pilot Study (n=11)	As-Needed (n=16)	Scheduled (n=15)	All Patients (n=31)	Combined (n=42)
Baseline	4.00 (2.19)	4.69 (2.47)	4.00 (1.25)	4.35 (1.98)	4.26 (2.01)
End FabAV infusion	3.00 (1.34)	3.31 (1.30)	3.20 (1.42)	3.26 (1.34)	3.19 (1.33)
Hour 1	2.91 (1.04)	3.19 (0.91)	3.07 (1.33)	3.13 (1.12)	3.07 (1.09)
Hour 4 or 6	2.40 (1.07)	2.63 (1.31)	2.60 (1.45)	2.61 (1.36)	2.56 (1.29)
Hour 12	2.55 (1.21)	2.38 (1.20)	2.40 (1.12)	2.39 (1.15)	2.43 (1.15)

*Mean (SD).

Figure 2.
Course of SSS by individual component scores in combined pilot and randomized comparative trials of Crotalidae Polyvalent Immune Fab (Ovine).



An important and unexpected observation of these trials was the phenomenon of recurrence. Recurrence was defined as the return of any venom effect after documentation that the abnormality had resolved. Although FabAV successfully achieved initial control of the envenomation syndrome in all patients, there were patients in whom recurrence of venom injury was discovered. The return of progressive swelling after initial control was described as a *local recurrence*, whereas the term *coagulopathy recurrence* described the return of thrombocytopenia, hypoprothrombinemia, prolongation of prothrombin time, or elevation of fibrin split products after initial control had been achieved. Details of recurrence phenomenon are included in 2 other articles from this symposium.

Recurrence may appear to be a new phenomenon, but has been described previously after the use of antivenoms in many countries, including the United States.[29–31] In addition, Dart[6] and Bogdan et al[32] retrospectively applied this definition of recurrence to the records of 354 patients from a North American snake envenomation database. There were 112 patients for whom a coagulopathy was reported in the medical record (defined as ≥1 abnormalities of platelets, prothrombin time, or fibrinogen level). To be included in the analysis, the initial administration of ACP had to result in the return of coagulation test results to the normal range (otherwise, the definition of recurrence could not be fulfilled). Surprisingly, the treating physician repeated the coagulation test in only 31 of the patients with an initial coagulopathy. In most cases, therefore, it was unknown whether the coagulopathy resolved or recurred after treatment with ACP. However, among the 31 patients in whom repeat coagulation tests were performed, 14 (45%) had a recurrence of their coagulopathy. The recurrent coagulopathy appeared serious in 2 cases: 1 patient with a fibrinogen level of 29 mg/dL and 1 with a platelet count of $36 \times 10^3/mm^3$. This study was limited by the retrospective data collection and the infrequent testing for recurrence. Nevertheless, it demonstrates clearly that coagulopathy may recur after initially successful use of ACP.

SAFETY OF ANTIVENOMS

The infusion of animal serum–derived pharmaceuticals can produce severe adverse reactions ranging from a rash

Table 3.
Literature summary for acute reactions and serum sickness associated with ACP.

Description	Patients	Result	Reference
Class I evidence—none			
Class II evidence			
Observational trial	17 patients received ACP: 8 had follow-up Ages ranged 8–38 years, 6 were male	6/8 (75%) developed signs of serum sickness	34
Class III evidence			
Chart review	100 patients treated with 2–23 vials of ACP	75% of patients who received >3 vials developed serum sickness	35
Chart review	36 patients, ages 2–71 years: 22 patients received ACP	4 (18%) patients developed serum sickness	36
Chart review	56 patients: 33 were treated with antivenom	8 patients (24.2%) had an acute allergic response; 5 patients (15%) developed serum sickness	37
Chart review	40 patients, 26 treated with ACP	Acute reactions in 6 (23%) of 26 evaluable patients, including 3 with hypotension; serum sickness occurred in 10 (50%) of evaluable patients	17
Chart review	37 children, ages 1–19 years	1 patient developed anaphylactic shock and was treated with epinephrine	38
Chart review	29 children, ages 9 months–7 years; 7 were treated with ACP	Acute reactions not reported; all 7 (100%) patients developed serum sickness	20
Chart review	67 patients: 5 unidentified; 23 patients received ACP	Acute reactions occurred in 13 (56%) patients; of these, 9 had severe anaphylaxis with shock symptoms	39
Chart review	227 patients, ages 1–83 years, 93% male; 211 treated with ACP	Acute reactions occurred in 51 (25%) of 207 patients; 1 patient had urticaria and bradycardia, serum sickness was reported in 10 (11%) of 94 patients	18

Multiple individual case reports document the association of acute reactions and serum sickness with the administration of ACP, but are not detailed here.
Class I evidence: properly controlled randomized and blinded clinical trials, Class II: prospective, nonrandomized or nonblinded clinical trials, cohort or well-designed case-control studies, dramatic results in uncontrolled studies and volunteer studies, Class III: retrospective case series, case studies.

to death. Serum reactions may either develop acutely during the infusion, such as in anaphylaxis or anaphylactoid reactions, or they may be delayed for several days, as in the case of serum sickness. Serum sickness is a clinical syndrome that involves fever, diffuse rash, intense urticaria, arthralgia, hematuria, and constitutional symptoms that persist for several days. Serum sickness often requires symptomatic therapy with antihistamines and systemic administration of steroids and may temporarily disrupt patients' activities such as the ability to work.

Because it is a partially purified product of horse serum, ACP retains large amounts of proteins that do not neutralize venom.[33] There are numerous case reports that document acute reactions (urticaria, bronchospasm, hypotension) during infusion of ACP (Table 3). In addition to case reports, retrospective series report incidence rates for acute reactions ranging from 23% to 56% (Table 3). It is also widely believed that ACP has caused death from anaphylaxis. We are aware of at least 3 deaths that appeared to be caused by anaphylaxis induced by ACP; however, there seem to be no such cases published in the medical literature.

In contrast to ACP, the overall incidence of acute reactions for the FabAV was 6 (14.3%) of 42 patients. Nearly all of the events were mild or moderate (Table 4). The most severe case was easily treated with the typical measures. Because data collection was prospective in the FabAV trials, but retrospective in studies of ACP and therefore more likely to detect adverse events, it appears likely that FabAV causes acute reactions less frequently than ACP. Anaphylaxis has not occurred with FabAV and its safety profile is promising; however, it should be realized that ACP has been used in thousands of patients and thus its adverse effects have been described more thoroughly. Experience with FabAV is too limited to conclude that anaphylaxis will not occur.

Serum sickness is a common complication of ACP, although the data are limited to 1 prospective study, several case reports, and retrospective case reviews. Conventional teaching is that serum sickness develops in all patients who receive a dose of ACP greater than 5 vials; however, the published literature does not support this estimate. The only prospective study of ACP reactions found that serum sickness developed in 6 of 8 evaluable patients.[34] In a retrospective case review, Corrigan et al[35] found that serum sickness occurred in 75% of patients treated with 3 or more vials of ACP, and Jurkovich et al[17] reported serum sickness in 86% of patients treated with 8 or more vials of ACP. However, other investigators report rates as low as 15% (Table 3).

In contrast, the overall rate of serum sickness after administration of FabAV was 16% (6/38 patients) in the 2 trials.[27,28] The investigator classified the severity of illness as mild or moderate in all cases, and only 4 of the patients received symptomatic therapy for serum sickness. Of note, 5 of the reactions were associated with 1 early lot of study antivenom, which was later discovered to have been incompletely purified. The error allowed retention of Fc in the product. If the patients receiving this lot of FabAV are omitted from the calculation, the overall incidence is 3% (1/30 patients).

USE OF ANTIVENOMS

Regardless of the specific antivenom chosen, the decision to administer antivenom involves a risk/benefit analysis. An effective antivenom would terminate or prevent the

Table 4.
Adverse events during infusion of Crotalidae Polyvalent Immune Fab (Ovine).

Patient No.	Severity	Description	Dosing Group	Treatment
1	Mild	Isolated urticaria	As-needed	None, resolved spontaneously
2	Mild	Isolated urticaria	As-needed	None, resolved spontaneously
3	Mild	Isolated urticaria	Scheduled	Diphenhydramine, cimetidine, methylprednisolone
4	Mild	Isolated urticaria	As-needed	Cimetidine, methylprednisolone
5	Moderate	Allergic reaction (cough and urticaria) in patient with history of reactive airway disease	Scheduled	The infusion was stopped. The patient's symptoms responded promptly to diphenhydramine and ranitidine. The reaction recurred when the infusion was restarted. No further antivenom was administered.
6	Moderate	Allergic reaction—hives, dyspnea, and wheezing in patient with history of reactive airway disease	Scheduled	The infusion was stopped. The patient's symptoms responded promptly to diphenhydramine, famotidine, and albuterol therapy. Additional antivenom was infused without further reaction using the precaution of an epinephrine infusion.

effects of pit viper venom: local injury (eg, swelling, ecchymosis), coagulopathy (eg, thrombocytopenia, hypoprothrombinemia, prolongation of international normalized ratio), and systemic (eg, hypotension).

The indications for administration of crotaline snake antivenom have not been rigorously defined. However, the medical literature indicates that the most common indications appear to be progressive venom injury, such as worsening local injury (swelling or ecchymosis), a clinically important coagulation abnormality, or systemic effects (hypotension, altered mental status). Administration of antivenom soon after envenomation would be expected to reverse some manifestations of envenomation such as hypotension and coagulopathy and prevent further progression of local manifestations.

The correct dosage of ACP is difficult to determine as there are no prospective studies. The package insert recommends 2 to 4 vials for a minimal envenomation, 5 to 9 vials for a moderate envenomation, and 10 to 15 vials or more for a severe envenomation.[40] The need for additional ACP is based on the clinical response to the initial dose and continuing assessment of the severity of poisoning. If swelling continues to progress, if systemic symptoms or signs of envenomation increase in severity, or if new manifestations appear, administration of an additional 1 to 5 vials is recommended.[40]

FabAV is administered in a different manner. Whereas ACP is titrated to the grade of envenomation (dose→reassess→dose→reassess), the studies of FabAV use the concepts of initial control and subsequent scheduled therapy. First, a large dose of FabAV is administered to achieve initial control. Initial control is defined as cessation of progression of all components of envenomation: local effects, systemic effects, and coagulopathy. In the patients studied to date, a total of 3 to 12 vials of FabAV have been administered to establish initial control. After control has been established, additional 2-vial doses are infused at 6, 12, and 18 hours. The regimen is based on the randomized comparative trial of 2 different dosage schedules.[28] After initial control was achieved with 6 to 12 vials, patients were randomly assigned to 2 groups: the scheduled dosage group (2-vial doses were administered every 6 hours for 3 doses), or the as-needed dosage group (no additional 2-vial doses were administered unless the investigator judged the patient's condition to have worsened). Overall, patients in both groups improved and their SSSs were not statistically different (Table 2). However, 8 of 16 patients in the as-needed dosage group required treatment with additional (unplanned) antivenom doses for recurrent venom effects, whereas none of the patients in the scheduled dosage group received additional doses outside the protocol (P=.026). In essence, the patients in the as-needed group required additional antivenom in many cases and their dosage schedule became similar to the scheduled group. Further, those in the scheduled group had fewer episodes of coagulopathy recurrence. Therefore, use of the scheduled dosing regimen is recommended in the product labeling.

A troubling characteristic associated with the administration of all antivenoms provided in dry form involves solubilization (reconstitution). Both ACP and FabAV are provided as lyophilized powders. Once the decision has been made to treat a patient with antivenom, any time lost to antivenom preparation permits undesired deterioration of the patient's condition. For example, a delay of an hour needed to reconstitute ACP can be life-threatening in a rapidly progressive envenomation. During the clinical trials of FabAV, investigators were asked to document reconstitution times. All vials were judged to be ready for infusion within 30 minutes (<10 minutes in 12 [31.5%] patients, 10 to 20 minutes in 14 [36.8%] patients, and 20 to 30 minutes in 12 [31.5%] patients; data were not recorded for 4 patients).[28] Further, Hill et al[41] compared the time of reconstitution of ACP and FabAV. In a randomized blinded trial, they studied the subjective assessment of when medical clinicians decided a vial of antivenom was ready for administration. The median time for ACP was greater than 90 minutes compared with 40 minutes for the FabAV.

After reconstitution, an antivenom must be infused. Infusion rate is believed to play a role in the rate of anaphylactoid reactions. However, there are again no human experimental data available to guide administration of ACP. The package insert for ACP recommends intravenous administration after dilution of the total dose in a crystalloid solution using an antivenom/diluent ratio of 1:1 to 1:10. An initial 5- to 10-mL aliquot of ACP is infused over a 3- to 5-minute period with careful observation for evidence of anaphylaxis. If no symptoms or signs of an immediate systemic reaction appear, the infusion is continued at "…the maximum safe rate for intravenous fluid administration."[40] In the clinical trials, the initial dose of FabAV was diluted to a volume of 250 mL in a crystalloid fluid and the total dose infused intravenously over 1 hour.[28] The solution was infused slowly for the first 10 minutes and the dose increased to complete the infusion within 1 hour.

Another discovery during research on the FabAV was its durability. Heat and motion studies indicate that the antivenom is resistant to a remarkable range of temperature and handling conditions.[42] Using an animal lethality model, Decker et al[42] demonstrated no loss of efficacy when

FabAV was stored at 50°C (122°F) for 60 days. There was no loss in potency in this model until the drug was exposed to 70°C (158°F) for at least 30 days. Additionally, FabAV was stable despite continuous tumbling in a rock polisher for 30 hours. These are the first data indicating that antivenoms would be expected to be stable under conditions involved in wilderness conditions.

In summary, the use of antivenoms remains a complex medical procedure. The dynamic and erratic course of snake envenomation and the imperfect treatments available create an environment requiring close patient monitoring and careful decisionmaking. Consultation with a physician experienced in the treatment of snakebite is recommended for most cases. There are now 2 alternatives for antivenom treatment. It is likely that both products are effective, although prospective evidence is available for only Crotalidae Polyvalent Immune Fab (Ovine). Neither product has been tested in the treatment of copperhead snakebite. Safety data indicate that the new product likely has an improved safety profile; however, only clinical experience will determine the extent of this improvement under conditions of clinical use.

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"The Pink Sheet"®

PRESCRIPTION PHARMACEUTICALS AND BIOTECHNOLOGY

THE NEWS THIS WEEK

Vol. 63, No. 20 May 14, 2001

FDA Approves *Gleevec, Campath* On Eve Of ASCO

- **NOVARTIS *GLEEVEC* FOR CML WILL BE AVAILABLE IN PHARMACIES BY MAY 18,** in time for oncologists returning from ASCO meeting. FDA approves oral leukemia drug in two-and-a-half months, notes further studies needed to evaluate improved survival benefit and early-stage disease therapy 3

- ***GLEEVEC* WILL COST PATIENTS $2,000-$2,500 A MONTH,** comparable to other chemotherapy and interferon therapies, company says. Patient assistance program will ensure patients pay no more than 20% of annual income for the drug. Pending legislation would extend Medicare coverage to Gleevec 4

- **BERLEX *CAMPATH* STUDY IN COMBINATION OR SEQUENTIAL USE WITH *FLUDARA*** could begin later this year following accelerated approval of monoclonal antibody for third-line chronic lymphocytic leukemia treatment May 7. Confirmatory study on clinical benefit versus alkylating agent to be completed by 2006 5

- **Medicare "reform" definition emerging as obstacle to Rx benefit, Hill staffers** say during Schwab WRG conference. Too much reform will block drug benefit, Democratic staffer says. Administration remains committed to tying reform to drug benefit. Rep. Thomas is happy to be Medicare reform "colonel," following Administration's lead 22

- **Waxman/Hatch bills could follow reimportation model in Congress, GPhA's Nixon suggests:** changes to generic approval procedures could gain momentum if work on Medicare Rx is held up. Reform bills should await FTC survey on patent settlements, Hatch aide says. Hatch is more interested in intellectual property for new technology than in "tinkering." Patent hearings in Senate Judiciary and Commerce Committees are postponed 18

- **Pediatric labeling negotiations would be limited to six months or face public review** under Senate pediatric bill. Proposal would award contracts to outside researchers when manufacturers decline to study off-patent products 21

- **FDA antihistamine switch review moves into legal phase after advisory committee supports** OTC safety of *Claritin*, *Allegra* and *Zyrtec* in response to petition from Wellpoint, and over opposition of manufacturers 34

Novartis Wants Roche To Stay Put: Is Merck Ready To Move?

- **Novartis sets up neighborhood watch: $2.8 bil. stake in Roche will allow company to monitor** strategic moves by its Swiss neighbor. Novartis buys 20% of Roche voting shares from disaffected shareholder, keeping options open in merger and acquisition field. Will deal give Novartis some access to Genentech pipeline? 11

- **Merck tries Rosetta as stepping stone: $620 mil. acquisition will boost Merck's genomics presence** and could increase its tolerance for still-larger deals. Biotech deal is announced against backdrop of published reports that Merck is considering $90 bil. deal for Schering-Plough 10

- **Carter-Wallace *Felbatol* successor is one wild card in sale of healthcare business** to investment group led by former Warner-Lambert exec Anthony Wild. Flurofelbamate is nearing IND stage. Carter-Wallace will join growing ranks of specialty pharma companies operating in gaps between larger firms 13

"The Pink Sheet" (ISSN 1530-6240) is published weekly (except the week of Christmas) by F-D-C Reports, Inc., 5550 Friendship Blvd., Suite One, Chevy Chase, MD 20815-7278. The annual subscription rate is (1) $1,200 or (2) $665 for additional copies mailed in the same envelope with $1,200 subscription. Cole Palmer Werble, President. Periodicals Postage paid at Bethesda, MD. POSTMASTER: Send address changes to "The Pink Sheet", 5550 Friendship Blvd., Suite One, Chevy Chase, MD 20815-7278.

Wyeth To Discontinue *Antivenin;* Protherics *CroFab* Production Increasing

Protherics expects enough supply of rattlesnake antivenom ***CroFab*** to meet extra demand caused by a shortage of Wyeth's ***Antivenin*** (crotalide).

Protherics has been stepping up production of CroFab since its launch in December in time for the snake-bite season, the company said.

CroFab is the only alternative to Wyeth's Antivenin in the U.S. The product was approved Oct. 2 while Antivenin production was halted. Savage distributes CroFab for Protherics ("The Pink Sheet" Oct. 9, 2000, p. 17).

Wyeth informed FDA at the end of last year that it would cease Antivenin production by the end of 2001, the company said. The company informed customers about the decision in January. The notification may have resulted in increased Antivenin demand due to stockpiling, the company said.

Wyeth announced May 10 that it would experience an Antivenin shortage from May to late June. The company attributed the shortage to the increased demand and "manufacturing limitations" at its Marietta, Penn. plant.

Wyeth contacted Savage about the shortage at the end of March. Savage obtained usage data on Antivenin from FDA, and adjusted production estimates accordingly, the company said.

Protherics, which sets its manufacturing capability according to the forecasts from Savage, expects to build capacity to cover market needs once Wyeth discontinues Antivenin production.

Before it discontinues Antivenin operations, Wyeth estimates that it will produce a year's supply of rattlesnake Antivenin. The company also expects to produce five years worth of coral-snake antivenom by the end of the year.

Wyeth will continue to produce Antivenin at normal capacity through the end of the year, Wyeth said.

Last year, Wyeth had controlled the distribution of Antivenin inventory in order to maintain supply through the rattlesnake season, which lasts from April through October. Wyeth had ceased production at Marietta from December 1999 to December 2000.

Production of Antivenin resumed shortly following a consent decree by FDA in October ("The Pink Sheet" Oct. 9, 2000, p. 3).◆◆

FDA Recalls & Court Actions — May 9, 2001

CLASS II

Lilly *Keftab*

Cephalexin hydrochloride, 500 mg. NDC 5147-9034-02, 6445-034-02, 64455-034-01 and 64455-034-10. Keftab tablets are sold in bottles of 100 and 60 count blisters. Lot no./Exp.: 3AP73A, 10/1/01; 3AR48A, 1/1/02; 3AS36A, 2/1/02; 3AS37A, 8/1/02; 3AD93A, 4/1/01; 3AD94A, 4/1/01; 3AD96B, 4/1/01; 3AE63B, 4/1/01; 3AG03A, 5/1/01; 3AG38A, 4/1/01; 3AG40A, 10/1/00; 3AG73A, 5/1/01; 3AH64A, 8/1/01; 3AH65A, 2/1/01; 3AM54A, 10/1/01; 3AM55A, 10/1/01; 3AP74A, 10/1/01; 3AR46A, 12/1/01; 3AR47A, 6/1/01; 3AR49A, 1/1/02; 3AR50A, 1/1/02; 3AS34A, 2/1/02; 3AS35A, 2/1/02; 3AS36A, 2/1/02; 3AS37A, 2/1/02; 3AS38A, 2/1/02; 3AS39B, 8/1/01; 4AA77A, 2/1/02; 4AA78A, 8/1/02; 4AD08A, 5/1/02; 4AD09A, 11/1/01; 4AD10M, 12/1/01; 4AE21A, 6/1/02; 4AE22A, 12/1/01; 4AE23M, 1/1/02; 4AE24M, 2/1/02; 4AE25B, 2/1/02; 4AE26B, 8/1/02; 4AF21D, 8/1/02; 4AF22A, 8/1/02; 4AF23A, 8/1/02; 4AF24A, 8/1/02; 4AG82A, 3/1/02; 4AG83D, 9/1/02; 4AG84M, 3/1/02; 4AK36C, 3/1/02; 4AK37A, 9/1/02; 4AK38B, 9/1/02; 4AK39A, 9/1/02; 4AK40A, 10/1/02; 4AK41B, 10/1/02; 4AK42B, 4/1/02; 4AK44C, 4/1/02; 4AL40A, 8/1/02; 4AM60A, 9/1/02; 4AN34B, 11/1/02; 4AN35A, 11/1/02.

Manufacturer: Lilly del Caribe, Carolina, PR.

Recalled by: Eli Lilly, Indianapolis, Ind., by letters dated 3/27/0. Firm-initiated recall complete.

Distribution: Nationwide; 45,189,243 units.

Reason: Problems with dissolution properties.

Recall number: D-191-1.

REVIEW ARTICLE

Toxicol Rev 2005; 24 (4): 217-227
1176-2551/05/0004-0217/$34.95/0

Treatment of US Crotalidae Bites

Comparisons of Serum and Globulin-Based Polyvalent and Antigen-Binding Fragment Antivenins

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Contents

Abstract

In the US, two antivenins are marketed for the treatment of snake envenomation. The horse-derived serum-globulin-based Antivenin (Crotalidae) Polyvalent (ACP) has been available since 1954. There are few data on the efficacy and incidence of adverse events that occur following the administration of ACP. Most of the data are retrospective, anecdotal, or case reports. In 2000, ovine-derived serum-globulin-based ACP (Crofab®) became available. Crofab® is said to cause fewer reactions than ACP, but there are few comparative data to

substantiate this claim. Although both antivenins ameliorate the systemic symptoms following snake envenomation, the efficacy of either antivenin in decreasing oedema and swelling is unknown for a number of reasons. Clinical trials are small and have not included control arms. The degree of oedema, as well as the efficacy of the antivenin in decreasing oedema, may depend on the genera of the snake (usually unknown) that envenomated the patient. This article compares available data on clinical aspects of the two antivenins. More prospective data are needed to determine the comparative efficacy of the two antivenins, or the efficacy of Crofab® in preventing tissue oedema. There are still unanswered questions regarding the optimal dosing regimen of Crofab®.

Approximately 98% of snake envenomations in the US are caused by snakes belonging to the Family Viperidae (previously designated Family Crotalidae), specifically from snakes in the Subfamily Crotalidae. Otherwise known as 'pit vipers', the Crotalidae includes the three genera: *Crotalus* (rattlesnakes), *Sistrurus* (massasauguas or pigmy rattlesnakes), and *Agkistrodon* (copperhead and cottonmouth).[1]

In 1954, Wyeth Pharmaceuticals began production of serum-globulin-based Antivenin (Crotalidae) Polyvalent (ACP). Although a significant number of anaphylactoid reactions and serum sickness followed ACP administration,[2,3] it was the only available pharmacological treatment for serious Crotalidae envenomations. No prospective human clinical trials were done comparing ACP to supportive care.

In October 2000, the US FDA approved ovine-derived serum-globulin-based ACP (Crofab® [1]; Crotalidae Polyvalent Immune Fab [ovine]) for the treatment of minimal and moderate North American Crotalidae envenomations. This antivenin is manufactured using a complex purification process and appears to have fewer adverse effects than ACP. This article compares these two antivenins.

1. Manufacturing of Antivenin (Crotalidae) Polyvalent (ACP) and Antigen-Binding Fragment Antivenom (FabAV)

ACP is a refined and concentrated preparation of serum globulins obtained by fractionating blood from healthy horses immunised with venom from: *Crotalus adamanteus* (Eastern diamond rattlesnake), *C. atrox* (Western diamond rattlesnake), *C. durissus terrificus* (tropical rattlesnake, Cascabel), and *Bothrops atrox* ('Fer-de-lance'). These snakes were chosen because they contain most of the antigens found in pit vipers throughout the world. The product is standardised by the dose rcquired to prevent lethality following multiple intravenous administrations of venom in mice.[4]

Antigen-binding fragment antivenom (FabAV) [manufactured by Protherics Inc., Brentwood, TN, USA] is produced by immunising sheep with one of four Crotalidae snake venoms (*Crotalus atrox, C. adamanteus, C. scutalatus* and *Agkistrodon piscivoris*). Ovine immune serum from each flock is digested with papain to produce univalent Fab fragments that are separated during the purification process from the more immunogenic fragment crystallisable (Fc) portion of the antibody. The four individual monospecific Fab preparations are combined to achieve a specific minimum potency per vial for each venom component.[5]

2. ACP

2.1 Efficacy of ACP

There are no controlled prospective human studies demonstrating the efficacy of ACP compared with placebo. In mice, ACP halts myonecrosis, reverses haemorrhage and decreases lethality, although efficacy diminishes if administration is delayed.[4] In rabbits, ACP improves survival and preserves muscle mass and function when compared with fasciotomy and debridement.[6] In dogs, ACP decreases intracompartmental pressure compared with controls.[7] Based on animal studies, retrospective studies and case reports, ACP appears to reverse the life-threatening and coagulopathic manifestations of Crotalidae envenomation.[4,8]

2.2 Indications and Dosing Regimen for ACP

The severity of the envenomation determines the antivenom dose. Since ACP is significantly immunogenic, its risks and benefits must be considered before initiating therapy.

Envenomation grading systems based on clinical signs and symptoms have been proposed by Russell et al.[9-11] and others.[12-14] The grading system used clinical parameters to determine the severity of the bite and the dose of antivenin and gained some acceptance in clinical practice.[2,11-14] A validated severity score was not utilised in determining these recommendations, nor were the recommendations subsequently validated in any clinical trial. However, these recommendations were frequently the basis

1 The use of trade names is for product identification purposes only and does not imply endorsement.

Table I. Grading envenomation severity

Grading classification	Grading definition	Clinical findings	Antivenin recommended?
0	Absent or minimal envenomation	There is no evidence of envenomation, but a snakebite is suspected. A fang-inflicted wound may be present. There is minimal pain and <2.5cm of surrounding oedema and erythema. No systemic manifestations or laboratory abnormalities occur during the first 12h after the bite	No
1–2	Minimal envenomation	Snakebite is suspected. A fang-inflicted wound is usually present. There is moderate pain or throbbing localised at the fang wound(s), surrounded by 2.5–12.5cm of oedema and erythema. No systemic or laboratory abnormalities are present after 12h of observation	No
2	Moderate envenomation	Pain, oedema spreading toward the trunk, and petechiae and ecchymoses are present but limited to the area of oedema. Nausea, vomiting, giddiness, and a mild temperature elevation may be present	Yes, 5 vials
3	Severe envenomation	The envenomation appears minimal to moderate initially, but the clinical course is rapidly progressive. Oedema spreads rapidly and petechiae and ecchymoses may be present. Varying degrees of coagulopathy, tachycardia, hypotension, and a subnormal temperature may occur	Yes, 5–10 vials
4	Severe envenomation	Local manifestations include sudden pain, rapidly progressive swelling that may reach and involve the trunk within a few hours, ecchymoses, bleb formation and necrosis. Systemic manifestations, often commencing within 15 min of the bite, include weakness, nausea, vomiting, vertigo, and numbness or tingling of the lips or face. Muscle fasciculations, painful muscular cramping, pallor, sweating, cold and clammy skin, rapid and weak pulse, incontinence, convulsions and coma also may be observed. Death may occur	Yes, ≥10 vials

for the administration of ACP. Table I is a synthesis of the published grading systems.[9-14]

2.2.1 Children

There are some unique considerations with paediatric patients. The dose of antivenin should be determined by the severity of signs and symptoms (degree of envenomation) rather than the bodyweight of the patient.[15] Volume overload must be avoided, but administering antivenin as a concentrated solution may increase the incidence of anaphylaxis. Following rattlesnake envenomation, 18 paediatric patients received ACP doses based on the severity of the bite. There were no adverse events.[16] Seventy-five vials of ACP were administered to a child with systemic manifestations of envenomation without adverse event. She did develop serum sickness.[17] In another report, seven children received ACP without adverse event. All seven developed serum sickness within 10 days of treatment.[18]

The need for additional ACP in any patient is based on the clinical response to the initial dose and the continuing signs and symptoms of envenomation. If swelling continues to progress, if systemic symptoms or signs of envenomation increase in severity, or if new manifestations of envenomation appear, five to ten more vials should be administered. There is not a maximum dose. The total required dose is the amount needed to reverse or stabilise the toxic manifestations of the venom as determined by the clinical picture.[14-17]

2.3 Contraindications (ACP)

Following Crotalidae envenomation, the patient history should include questions regarding known allergy to horse by-products, horse serum, prior horse serum administration and the presence of asthma, hay fever, or urticaria. Patients with this history may be at a greater risk of developing an anaphylactoid response to ACP. There are no absolute contraindications to ACP administration, although this has not been clinically evaluated. Based on trials with small patient numbers, skin testing does not predict which patients will have an anaphylactic reaction. In one study, four of six patients with positive skin tests and two of 20 patients with negative skin tests developed immediate anaphylactoid reactions to ACP. ACP administration was deemed necessary in three of the six patients developing a reaction. Subsequent ACP administration was tolerated (with concurrent antihistamine [three of three patients] and epinephrine [two of three patients] administration) in all three patients.[19]

2.4 Follow-Up of ACP-Treated Patients

Following hospital discharge, there is no consensus regarding timing of follow-up of envenomated patients who have been treated with ACP. There is evidence that coagulopathy may recur after normalisation with ACP administration.[20] Although this recurrence is infrequently addressed in the literature, it may be that the observation was recent and subsequent studies have not further addressed the finding due to the availability of a new antivenin. It seems reasonable to re-evaluate patients within a week and check coagulation parameters, particularly if the patient had signs of coagulopathy while in the hospital. Patients should be instructed to return if symptoms of serum sickness characterised by fever, malaise, urticaria, lymphadenopathy, rash or arthralgia, occur. Onset of serum sickness is usually 1–2 weeks following the administration of antivenin.[14,21]

2.5 Immediate and Delayed Reactions Following ACP Administration

2.5.1 Immediate Reactions

Immediate hypersensitivity reactions can be divided into anaphylaxis or anaphylactoid. Anaphylaxis occurs when re-exposure to an allergen causes cross-linking with IgE on mast cells and basophils causing degranulation and release of histamine and other inflammatory mediators. Anaphylactoid reactions are not IgE-mediated, and do not require previous sensitisation but also release inflammatory mediators from mast cells and basophils. The signs and symptoms of these two reactions may be indistinguishable. These include urticaria and pruritis, laryngeal oedema, wheezing, nausea, vomiting, diarrhoea, hypotension, bradycardia, cardiovascular collapse, loss of consciousness and possibly death.[21]

Due to the lack of formal studies, the actual incidence of ACP-induced reactions is unknown. In one study, during the administration of ACP for systemic symptoms of envenomation, 13 of 25 patients developed severe anaphylaxis and shock. There were no deaths.[22] In another study of 112 patients receiving ACP, 26 had an acute reaction. None died and there were no sequelae.[23] In a retrospective review, 6 of 26 patients receiving ACP developed immediate hypersensitivity reactions; three developed cutaneous reactions, and three developed systemic signs of hypotension and dyspnoea. There were no deaths and no sequelae.[19]

2.5.2 Delayed Reactions

Serum sickness is caused by nonspecific deposition of immune complexes (IgG) in certain tissues. Immune complexes are usually cleared by the reticuloendothelial system, but may precipitate when there is increased vascular permeability and antigen excess. When immune complexes are trapped in the vascular wall with vasoactive mediators, complement is activated and further release of mediators results in serum sickness.[21]

In the previously discussed retrospective review of 26 patients treated with ACP (see section 2.5.1), 20 were available for follow-up. Ten of the 20 patients developed erythematous rash, pruritis, or urticaria 4–20 days after antivenin administration. All ten patients were treated with antihistamines: three were also treated with corticosteroids. The severity of symptoms of serum sickness delayed hospital discharge in two paediatric patients and required two other paediatric patients to be readmitted. All patients rapidly improved with no morbidity or chronic sequelae. Serum sickness occurred in 83% of those patients receiving more than eight vials of antivenin, and 38% in those receiving less than eight vials. Possibly due to the small sample size, this difference was not statistically significant.[19]

Serum sickness was subsequently reported in 102 of 181 (56%) patients receiving ACP for presumed rattlesnake envenomation. The frequency of serum sickness in patients who received <20, 20–29, 30–39, or ≥40 vials was 34%, 36%, 88% and 100%, respectively. Medications administered included prednisone (98%), antihistamines (92%), and histamine H_2 blockers (3%). Patients in this study were monitored until complete resolution of all symptoms.[3] Of 72 patients who received ACP for rattlesnake envenomation, 49 patients developed serum sickness. There was no morbidity or mortality. The incidence of serum sickness did not correlate with number of vials of ACP administered.[23]

In the available literature, there is a large variation in the reported frequency of both immediate and delayed reactions following ACP administration. Although the immediate reaction to ACP has life-threatening potential, there are very few deaths reported. Deaths are attributed to ACP administration[24,25] but the supporting documentation references articles written in the 1980s or earlier,[19,26,27] a time when the critical care of patients was much less sophisticated than now. Of those reported, it is difficult to discern if the death was a result of the envenomation or the antivenin administration. Although serum sickness is a concern, it does not appear to cause mortality or permanent morbidity.

3. FabAV

3.1 Efficacy of FabAV

Two studies have evaluated FabAV: (i) a prospective observational pilot trial of 11 patients;[24] and (ii) an open-label randomised comparative trial of two dosage regimens in 31 patients.[25] In both studies, inclusion criteria were: age of at least 10 years; a minimal or moderate Crotalidae envenomation (excluding copperhead envenomations); presentation within 6 hours of the bite; and

ongoing progression of local swelling or coagulopathy. Patients with a severe Crotalidae envenomation were excluded. Severe envenomation was defined as swelling, pain and ecchymosis involving more than an entire extremity; swelling threatening the airway; severe alteration of mental status; systolic blood pressure <90mm Hg; heart rate >150 beats/min; tachypnoea, respiratory insufficiency and abnormal coagulation parameters associated with clinical evidence of serious bleeding (not limited to minor haematuria, gum or nose bleeding).[24,25]

The patients in the two FabAV trials were evaluated using a snakebite severity score (SSS). Efficacy in both studies was defined as no further increase in the SSS. There was no control group. The SSS measured the severity of envenomation based on six organ systems: local wound (skin), pulmonary, cardiovascular, gastrointestinal, haematological and CNS. The only validation of the SSS was a retrospective review of 108 medical records of snakebite victims. Retrospectively, the SSS correlated well with the physicians' assessment of the patient's clinical condition at presentation ($p < 0.0001$, $r = 0.63$) and when the patient's condition was most severe ($p < 0.0001$, $r = 0.70$).[28] Although the SSS at the time of presentation to the hospital did not predict ultimate envenomation severity, this score was designed as a study tool, not as a clinical parameter to predict the need for or amount of antivenin.

In the observational trial of 11 patients, SSS improved in all patients following the infusion of 4–8 vials of FabAV. Two of the 11 patients developed re-emergence of limb swelling after initial improvement for several hours. Further antivenin was administered. Eight of the eleven patients had further follow up. One of the patients that did not return was contacted by telephone and had symptoms consistent with a late allergic reaction.[28]

In the open-label randomised comparative trial of two dosage regimens, each of the 31 patients initially received six vials of FabAV. This dose could be repeated once (for a total of 12 vials) at the clinician's discretion to achieve 'initial control', which was defined as the cessation of worsening of all evaluation measures. The primary assessment tool was the SSS. Efficacy was defined as a stable or decreasing severity score during the 12-hour evaluation period after establishment of initial control. A secondary measure, the investigator's clinical assessment, was used to confirm the severity score and ascertain its clinical relevance. Once initial control was achieved, patients were randomly assigned to one of two groups from a central call centre. Sixteen patients were randomised to receive antivenin as-needed (the PRN group) and 15 patients were randomised to receive scheduled antivenin. Patients in the PRN group received no further antivenin unless their clinical condition worsened, whereas patients in the scheduled group received the initial six vials and then an additional two vials of FabAV at 6, 12 and 18 hours. Initial control was achieved in all 42 patients with 3–12 vials of FabAV. Mean dose to achieve initial control was 8.7 vials.[25]

Although the total amount of antivenin administered was not statistically significantly different between the PRN and scheduled groups (a mean of 8.25 vials for the PRN group vs 7.4 vials for the scheduled group), recurrence of coagulopathy occurred more frequently in the PRN group. Eight of the 16 patients in the PRN group received additional doses for recurrence of local wound progression during the first 12 hours. At follow-up, 56% of the patients in the PRN group and 14% of patients in the scheduled group had a platelet count less than that at hospital discharge. 44% of the PRN group and 14% of the scheduled group had a fibrinogen level <50% of the level at hospital discharge. It should be noted that abnormalities were not associated with clinical signs/symptoms or evidence of bleeding.[25] These data constitute the basis for the recommendation that scheduled, rather than PRN, dosing be used.

It is interesting to note that more antivenin was required to achieve initial control in the patients randomised to the PRN group. This may be because the patients in the PRN group had more severe envenomations (100% moderately envenomated vs 87% moderately envenomated and 13% minimally envenomated in the scheduled group). This discrepancy causes one to question the conclusion that scheduled dosing is necessary. Even though the PRN group had slightly greater morbidity at the outset, there was no statistically significant difference in clinical severity scoring at any timepoint, despite the fact that they received 49% less antivenin after initial control was achieved.[25] It is difficult to assess the validity of the dosing recommendations due to the small number of subjects in the study.

In contrast to systemic symptoms, which improved over 12 hours in both groups, the local injury score did not improve in either group. This may be because local tissue destruction occurs in an irreversible manner. Thus, successful venom neutralisation may arrest progression, but would not improve local tissue destruction that had already occurred.[28]

3.2 Indications for FabAV

FabAV was approved by the FDA for the management of patients with minimal or moderate envenomation from North American crotalids. However, the indications for antivenin administration are still fairly ambiguous. Some authors argue that isolated coagulopathy is unlikely to cause life-threatening bleeding events[29] and that coagulation values predictive of major bleeding complications following crotalid envenomation have not been defined. Indications (although not validated) for antivenin admin-

istration based on the clinical trials include: clinically significant bleeding and any abnormal coagulation parameter, asymptomatic patients who develop multi-component coagulopathy that reaches critical values (e.g. international normalised ratio [INR] >3.0, activated partial thromboplastin time [aPTT] >50 seconds, fibrinogen concentrations <75 mg/dL, and platelet count <50 000/mm^3).[30] These same parameters have been suggested as guidelines to readminister antivenin to a patient whose coagulopathy initially improved and then recurred. However, validated criteria do not exist for repeat administration of FabAV following recrudescence. There is no evidence that treatment is required for recurrence of isolated coagulation abnormalities without clinically significant bleeding. Neither risk : benefit nor cost analysis have been performed.[31]

In the two studies of FabAV,[24,25] thrombocytopaenia in 42 patients improved initially with FabAV administration. However, the effect of antivenin on platelet count is inconsistent and may have to do with the species of snake and the timing of the thrombocytopaenia. Following timber rattlesnake envenomation, (a venom not used as an immunogen in either ACP or FabAV), a 38-year-old male's platelet count dropped from 157 000/m^3 on admission to 28 300/m^3 5 hours later. Although 46 vials of FabAV were subsequently administered for thrombocytopaenia, platelet counts continued to drop and reached 500/m^3 on day 3. Platelet count was 26 300/m^3 on hospital day 10 when he was discharged. There was no evidence of spontaneous bleeding. Platelet count remained depressed until 3 weeks after the envenomation when it rose to 255 000/m^3.[32] The expense associated with the administration of large quantities of either ACP or FabAV is significant. The average wholesale price of one vial of ACP is $US762.50 and the price of a two-vial pack of FabAV is $US2250.00 (2005 values).[33] Therefore, the FabAV procurement cost to treat the above patient was $US49 450. In another report, 14 vials of FabAV were administered to a patient to treat local signs and symptoms of envenomation from a red diamond rattlesnake *(Crotalus ruber ruber)*. However, due to persistent thrombocytopaenia (platelet count of 5000/m^3 on hospital day 4) he received 32 more vials of FabAV and ten vials of ACP. Neither antivenin seemed to affect platelet count.[34] The cost of treating thrombocytopaenia in this patient would be approximately $US42 025.

Although thrombocytopaenia appears to respond to antivenin administration in some reports,[35,36] other reports demonstrate resistance to antivenin therapy.[37-39] In animal studies and case reports, bone marrow biopsy during envenomation-induced thrombocytopaenia demonstrates normal marrow with adequate megakaryocytes.[39-41] These observations suggest that peripheral sequestration, not decreased marrow production is the cause of the early thrombocytopaenia. Antivenin may cause early improvement by reversing platelet aggregating venom constituents. However, late thrombocytopaenia may represent sequestration of platelets at the envenomation site of exposed endothelium and may not be reversed by antivenin.[34] That late thrombocytopaenia may not respond to antivenin should be considered when administering antivenin for this finding. Administration of a few vials may be warranted, but lack of response does not necessarily indicate the need for further antivenin at a very significant cost.

Although copperhead envenomations are common in the US, copperhead venom is not used in the preparation of FabAV. A retrospective chart review of 400 copperhead envenomations reported to the Carolinas Poison Center (Charlotte, NC, USA) identified 34 patients who received FabAV, the majority of whom had moderate envenomations. Initial response (defined as cessation of the progression of local tissue injury within 4 hours of FabAV administration) occurred in 28 cases. However, there were no standardised criteria or time intervals for assessing the progression of local tissue injury. Four cases were considered treatment failures, and recurrent swelling occurred in six patients. There was no control group, which is significant as tissue swelling following copperhead envenomations typically resolves spontaneously.[42]

As there were no controls in the clinical trials, it is difficult to determine indications for FabAV administration in patients with progressive local symptoms without coagulopathy or systemic signs/symptoms. Progressive local swelling may suddenly stop or improve without antivenin treatment. Natural progression of local effects is unknown due to lack of controlled trials. Nor are there data regarding indications or dosage of FabAV in severe envenomation as the clinical trials excluded patients with severe envenomation. Further investigations are warranted for both groups of patients.

3.3 Contraindications to FabAV

The manufacturer recommends that FabAV should not be administered to patients with a known history of hypersensitivity to papaya or papain unless the benefits outweigh the risks. Furthermore, as even sheep serum is immunogenic (even with the Fc component removed) the potential for allergic reactions must be considered prior to antivenin administration.[43]

3.4 Dosage of FabAV

The manufacturer recommends the administration of 4–6 vials of FabAV to achieve initial control (normalisation of the coagulation profile and stopping the progression of local soft tissue swelling); if initial control is not achieved, administer an addition-

al 4–6 vials; once initial control is achieved, administer two vials of FabAV at 6, 12 and 18 hours.[43]

This dosing regimen is based on the open label comparative dosage trial, and the impression that recurrent coagulopathy, soft-tissue swelling and late platelet/fibrinogen abnormalities occurred more frequently in the PRN group.[25,44]

3.4.1 Children

While specific studies in paediatric patients have not been conducted, dosage adjustment for age or weight is not recommended. Antivenom administration should be based on signs and symptoms of the patient that reflect venom load. The package insert recommends dilution of the antivenom in 250mL of normal saline to be administered over 1 hour. This volume is not a problem except for the very small patient. Successful administration of the standard adult antivenin dose in a child without complications or sequelae has been reported.[45]

3.5 Recurrent and Delayed Coagulopathy-Implications for the Follow-Up of FabAV-Treated Patients

The indications for antivenin administration in recurrent or delayed coagulopathy is difficult to discern as there is evidence that antivenin administration may not resolve late or recurrent isolated coagulopathy.

There is no available evidence that indicates that isolated or recurrent coagulopathy is clinically significant and needs to be treated.[24,25,46-48]

3.5.1 Recurrent Coagulopathy

Recurrent coagulopathy, defined as decreased fibrinogen, decreased platelets, or elevated prothrombin time (PT) after initial successful treatment with antivenin, occurred in patients in both clinical studies. In the observations study, there were three episodes of hypofibrinogenaemia, three episodes of thrombocytopaenia and one episode of an elevated PT. A typical example of the recurrent coagulopathy was a patient whose PT was 18.3 seconds (abnormal) on presentation, 25.7 seconds at the end of antivenin infusion, 22.0 seconds at 1 hour, 15.1 seconds at 4 hours, 13.6 seconds (normal) at 48 hours, but was 22.8 seconds (again abnormal) at 7-day follow-up.[24] Clinical bleeding did not occur in any patient.

One-half of the 31 patients had recurrent coagulopathy in the dosage study. However, no patient experienced significant spontaneous clinical bleeding. One patient with hypofibrinogenaemia developed minor bleeding after haemorrhoidectomy 12 days after envenomation. The fibrinogen level was undetectable and the haematocrit, which had been 36% on the second day following the bite, reached a low of 30%. Local bleeding was controlled with pressure, no blood products were administered, and the coagulation studies began to normalise within the next 25 hours.[25]

Of the 42 patients enrolled in the two studies, four withdrew within 4 days of study participation.[24,25] Twenty-nine of the 38 patients remaining developed coagulopathy (without clinical signs of bleeding) at some point during their course. Among the 29 patients with coagulopathy, 20 experienced late, persistent, or recurrent thrombocytopaenia or hypofibrinogenaemia. Sixteen of these patients were observed without further treatment. Coagulopathy in 12 of the 16 patients resolved spontaneously within 2 weeks. Four of the 16 patients had ongoing hypofibrinogenaemia (two with elevated PT) 14 days after enrolment. Of these four patients, one withdrew from the study at 1 week, and one was lost to follow-up. Two patients received additional FabAV at 6- and 9-day follow-up. Laboratory values were normal 2 weeks after their envenomations.[24,25] Patients who demonstrate a coagulopathy before therapy or during the first 12 hours after FabAV treatment have approximately a 66% chance of recurrence.[47]

The data suggest that the best indicator of late thrombocytopaenia or hypofibrinogenaemia is early thrombocytopaenia/hypofibrinogenaemia, respectively, and the degree of late decrease may correlate with the degree on presentation. Patients presenting with thrombocytopaenia or hypofibinogenaemia who are treated with FabAV should be re-evaluated within 5 days of initial therapy. Patients with an abnormal coagulation profile during the first 36 hours after envenomation should be reassessed 48 hours after antivenin administration.[47]

3.5.2 Delayed Coagulopathy

Patients who present with normal coagulation profiles may still develop late-onset coagulopathy. One patient with a Northern Pacific rattlesnake envenomation had normal laboratory values until 5 days after the bite. During the first 48 hours, she received a total of 18 vials of FabAV to treat local symptoms. On day 5, her fibrinogen was <50 mg/dL but platelet count and other coagulation studies were normal. No additional FabAV was administered. On hospital day 6, her fibrinogen remained <50 mg/dL, her INR was now 6.7, and aPTT was >250 seconds. There was no evidence of bleeding. She received an additional four vials of FabAV on hospital day 7. Platelet count and INR corrected but fibrinogen remained low at 94 mg/dL. By day 11, fibrinogen (as well as platelets and INR) were normal.[46]

A 40-year-old man with a northern Pacific rattlesnake envenomation was treated with FabAV. Oedema progression stopped after administration of 22 vials of FabAV within the first 48 hours of admission. However, he developed an INR >14.7 seconds, fibrinogen <50 mg/dL and platelets of 15 000/m^3 over the 7 days

following envenomation despite treatment with 32 vials of FabAV (ten more vials after the initial 22 vials during the first 48 hours). Coagulation abnormalities resolved between day 17 and 37. He did not experience clinical bleeding.[48]

3.6 Immediate and Delayed Reactions Following FabAV Administration

Six of the 42 study patients treated with FabAV experienced acute adverse reactions. Three patients were in the PRN group and three patients were in the scheduled group. Two resolved without treatment and four responded to histamine (H_1 and H_2) blockers and corticosteroids.[24,25]

Subsequent to the publication of these two studies,[24,25] acute urticaria, anaphylaxis, angioedema and delayed serum sickness, respectively, were reported in four patients following FabAV administration. A 36-year-old man bitten on his right middle finger by a small *C. viridis heleri* developed swelling, circumoral numbness and coagulopathy. Forty minutes after the initiation of antivenin, he developed generalised urticaria, angioedema of the oropharynx and tongue, wheezing, nasal congestion and cutaneous flushing. His oxygen saturation on room air decreased to 88%, his systolic blood pressure decreased 20mm Hg (92mm Hg), and his pulse rate increased to 136 beats/min. The infusion was discontinued. Administration of diphenhydramine 50mg (intravenous [IV]), famotidine 20mg (IV), a low-dose epinephrine infusion (IV), and inhaled salbutamol (albuterol) resulted in resolution of signs and symptoms within 20 minutes. Antivenin infusion was resumed and completed with no subsequent reactions. Epinephrine was discontinued 30 minutes after completion of antivenin administration. Four hours later, due to persistent swelling and defibrination, six additional vials of FabAV were administered concomitantly with low doses of intravenous epinephrine. No reaction occurred and he remained free of all sequelae at follow-up on days 2, 4 and 10 after envenomation.[49]

A 22-year-old hiker presented to the emergency department 3.5 hours after being bitten on her foot by an unidentified rattlesnake. Due to swelling to the ankle, INR of 1.3 and platelet count of 119/m^3, the decision was made to treat this patient with ACP despite a persistent wheal (>15mm in diameter) with surrounding flare at the skin testing site. After infusion of only 1mL of the antivenin solution, angioedema of her lips developed, she complained of nausea and began vomiting. The antivenin infusion was stopped, and epinephrine (0.3mL of 1 : 1000 subcutaneous [SC]) and methylprednisolone (125mg IV) were administered. Symptoms rapidly resolved. Her leg continued to swell, and arrangements were made to obtain FabAV. Seventeen hours after admission, oedema and induration had progressed to her knee, with tenderness into her groin. Her foot was ecchymotic, with multiple haemorrhagic bullae. Six vials of FabAV were diluted in 250mL of normal saline solution, and an infusion started at 30 mL/h and increased slowly to 250 mL/hour over a 25-minute period. When that infusion rate was reached, she complained of throat tightness, lip-tingling, shortness of breath, and exhibited generalised erythroderma, urticaria of extremities and trunk, and focal angioedema of her lips. Pulse oximetry and blood pressure remained normal. The antivenin infusion was stopped, diphenhydramine (50mg IV), famotidine (20mg IV), and methylprednisolone (125mg IV), as well as four separate doses of epinephrine (0.3cc SC) were administered over 30 minutes. Her erythroderma, urticaria and localised angioedema resolved over several minutes. Antivenin infusion was restarted and administered over 2.5 hours without event. The patient received two additional vials of FabAV every 6 hours for three more doses without further reactions, although she was pretreated with antihistamines before each infusion. By the fourth day, her swelling had improved, and she was discharged. In telephone follow-up 2 weeks after discharge, she had no symptoms compatible with serum sickness or bleeding. The lot number and expiration date of the FabAV vials administered to this patient were found to be identical to vials previously administered to another patient who experienced no adverse effects.[49]

In the 42 patients treated with FabAV in the prospective trials, seven patients experienced serum sickness (one documented via telephone).[24,25] One further case of delayed serum sickness has been reported.[49] All patients recovered without sequelae.

It is difficult to draw a conclusion regarding the indications and potential for immediate and delayed reactions following administration of FabAV. Numbers in the clinical trials are small. Snake bite can cause morbidity and mortality, yet so can antivenin administration. It is clear that antivenin should be administered for severe envenomation. What is more difficult to discern are the indications for FabAV in mild and moderate envenomations, isolated coagulopathy, or recurrent coagulopathy.

4. Pharmacokinetic Considerations

One of the reasons for recurrence of venom effects following Fab administration is a pharmacokinetic mismatch between antivenin and target venom components. Clearance of unbound antivenin is significantly faster than the clearance of some venom components.[50]

The distribution half-life of FabAV is approximately 2.5 hours. Clinically, signs and symptoms of local recurrence correlate well with the timing of the distributive phase when the circulating serum concentration of unbound FabAV falls. This indicates that

there may be a critical serum concentration above which progression of local injury into adjacent tissue is arrested. Repeated dosing during the first 25 hours increases the serum concentration and may prevent recurrence.[50]

Intramuscular deposition theoretically allows for sustained-release therapy. However intramuscular deposition of FabAV yields serum concentrations only 10% of those after IV administration, and fractional drug absorption is only 42% of that after IV administration, with significantly prolonged time to maximal plasma concentration.[51] These characteristics make intramuscular injection a poor choice for initial management, but the development of sustained-absorption vehicles may provide persistent plasma levels sufficient to prevent recurrent coagulopathy. Because of the longer half-life of ACP and because ACP distributes roughly to the vascular volume, a depot approach is unnecessary with ACP.

5. Antivenin Administration

The following are the authors' recommendations for antivenom administration based on available data.

5.1 ACP Administration

1. Ask about history of allergy (especially to horse serum products), asthma, hay fever, urticaria.
2. Skin testing is unnecessary as it is of no predictive value.
3. Determine initial dosage based on the clinical severity grading.
4. Start an intravenous infusion in two extremities. One line is for administering the antivenin. The second line is primarily for cardiovascular support (isotonic fluids, epinephrine, or antihistamines).
5. Diphenhydramine 50–100mg IV prior to antivenin infusion (elective).
6. Histamine H_2 receptor antagonist (e.g. cimetidine 300mg IV or ranitidine 50mg IV prior to antivenin infusion) [elective].
7. Add 10mL of diluent or normal saline to each vial. Mix by rolling between hands. Do not shake.
8. Inject 5–10 vials in 250mL. Administer intravenously at a slow rate initially (e.g. 10 mL/hour) and then progress to a faster rate (gradually increased over 30 minutes to 250 mL/hour) if no reaction occurs. Reduce volume of diluent in paediatric patients. Attempt to give total dose during first 1–2 hours.

5.1.1 Patients Who Develop an Acute Reaction During ACP Administration

1. Stop antivenin infusion.
2. If not already done, start a second intravenous line.
3. Epinephrine 1 : 10 000 constant infusion.
4. Diphenhydramine 50–100mg IV unless already given.
5. Histamine H_2 receptor antagonist IV unless already given.
6. Reconsider the need for antivenin. The primary considerations include the severity of the envenomation (life- or limb-threatening) and the seriousness of the reaction.
7. If antivenin is needed, due to life-threats or coagulopathy, restart the antivenin slowly and increase the rate of administration as tolerated by patient.

5.2 FabAV Administration

The manufacturer recommends that each vial of FabAV should be reconstituted with 10mL of sterile water (diluent not included) and mixed by continuous gentle swirling. The reconstituted 4–6 vials should be diluted further in 250mL of normal saline and administered within 4 hours. Skin testing is not required. FabAV should be initiated at a rate of 25–50 mL/hour over the first 10 minutes with observation for any signs and symptoms of allergic reaction. The infusion rate may then be increased to administer the first dose over an hour if no reaction occurs. If initial control is not obtained, infuse an additional 4–6 vials. Initial control is defined by complete arrest of systemic and local manifestations, and normal coagulation parameters.[43] The patient should be observed for at least 1 hour following the completion of antivenin administration. Once the initial envenomation symptoms have been controlled, additional two-vial doses of FabAV may be administered every 6 hours for 18 hours. Based upon the patient's clinical course and response to therapy, addition two-vial doses may be necessary.[52] Patients who develop an acute reaction during FabAV administration should be treated as described in section 6.1.1

6. Antivenin Availability

In May 2001, Wyeth Laboratories released an announcement warning of a transient supply outage for its antivenin. Currently, Wyeth Laboratories is producing and distributing their ACP product (Krenzelok EP, unpublished data). The bulletin cited manufacturing limitations and advised healthcare providers to contact Protherics to obtain FabAV if the Wyeth antivenin is not available.[53] Limitations in antivenin supply make it all the more important for practitioners to become familiar with both antivenins.

7. Costs

The packaging guidelines for FabAV advise administering 4–6 vials, which can be repeated once, to achieve initial control. Two vials are subsequently administered every 6 hours for a total of three additional doses. Thus, in the first 18 hours of treatment 10–18 vials of antivenin will be administered. A two-vial pack of FabAV costs $US2250 (2005 values). Therefore, the total drug

cost to the hospital for FabAV (10–18 vials) is $US10 750 (10 vials) to $US19 350 (18 vials). Direct patient charges will vary by hospital.[33]

In comparison, a moderate envenomation treated with ACP would initially receive 5–9 vials of antivenin. The total drug cost to the hospital for this therapy, based on an acquisition cost of $US762.50/vial, is $US3812.50–$US6862.60.[33]

Severe envenomations treated with 20–30 vials of ACP cost $US15 250–$US22 875 or treated with 34–47 vials of FabAV costs $US36 500–$US50 525.[33]

8. Conclusions

Will FabAV become the standard of care for all Crotalidae envenomations? While there are no prospective human investigations of ACP, there are decades of clinical experience with the drug. ACP is effective in the treatment of life-threatening or coagulopathic manifestations of Crotalidae snakebite.[2-11] Current literature alleges that the incidence of acute reactions to ACP administration is greater than the incidence of acute reactions to FabAV administration,[24,25] but we really do not know if this is the case. There are no comparative data and much of the data quoted are prior to 1990, when care in the intensive care unit was much less sophisticated.

FabAV is stated to halt the progression of local tissue destruction, reverse coagulopathy, and improve the parameters measured by the SSS in mild and moderate envenomations. However, there were no controls in this study and the assumption that the improvement in score was caused by the antivenin administration is based on the thought that progression would otherwise be expected.[25] Unfortunately, we do not know what the usual clinical course is for untreated snakebite with minimal to moderate envenomation. This is difficult to discern due to the variability in amount of venom injected, differences in first aid or other treatment, lethality of venom of different snakes, etc.

Recurrence of local swelling and isolated factor coagulopathy are common following FabAV administration. What we do not know is the incidence of isolated coagulopathy in untreated patients or in patients treated with ACP, although a retrospective review demonstrates that it occurs following ACP administration.[20] This recurrent coagulopathy may be a more common phenomenon that has not been previously reported until this study.[25] We do not know if we need to treat this recurrence, which is a costly undertaking.

It is difficult to compare the costs of ACP and FabAV. FabAV is approximately 2.8 times more expensive than ACP if patients receive standard doses. However, data from the existing literature comparing the two antivenins is dissimilar in both study design and in the severity of the envenomations. Ease of availability of FabAV may cause it to become the standard of care for Crotalidae envenomations. If this is the case, there are still many questions to answer regarding the indications for administration of this antivenin.

Acknowledgements

Edward P. Krenzelok has disclosed that he owns shares of Protherics, the manufacturer of FabAV.

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